Synthesis of Prodrugs of Anthracyclines and Evaluation of Their Use in Selective Chemotherapy

Een Wetenschappelijke Proeve op het Gebied van de Natuurwetenschappen

Proefschrift ter Verkrijging van de Graad van Doctor aan de Katholieke Universiteit Nijmegen, Volgens Besluit van het College van Decanen in het Openbaar te Verdedigen op Vrijdag 30 Januari des Namiddags om 13.30 Uur Precies

Door
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Geboren op 21 Januari 1966 te Nijmegen

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Voorwoord

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1 Introduction

1.1 General introduction

Approximately one person in five in the prosperous countries of the world dies of cancer and besides heart disease, this disorder is the main cause of death in the Western world.
In cancer, a mutation of a single healthy cell gives rise to selfish behavior of that cell. This endangers the whole multicellular organism while in healthy organisms, all cells collaborate and remain in their territories. When a mutated cell proliferates out of control, it will give rise to a tumor (a neoplasm). As long as the neoplastic cells remain clustered together and do not invade other tissue, the tumor is said to be benign (harmless). A complete cure can be achieved by removing the tumor surgically or by killing the tumor cells by irradiation. Neoplastic cells are said to be malignant if they have the ability to invade other tissue. Additionally, malignant cells can break loose to eventually enter the bloodstream to be transported to distant sites in the body and form a secondary tumor. This process is called metastazation and makes the disease especially hard to eradicate as the tumor cells remain no longer localized. A complete cure can no longer be effected by local therapies such as surgery or irradiation and a systemic therapy such as chemotherapy is required to treat patients with a malignant tumor.

1.2 Cancer chemotherapy

During World War I, mustard gas (sulfur mustard, chart 1.1) was used as an offensive weapon. Besides the use in chemical warfare, it was recognized that sulfur mustard had an effect on rapidly dividing cells of the gastrointestinal tract and on the blood forming organs. In the early literature [1] it is described that sulfur mustard could stop the development of chemically induced tumors in animals. After these experiments, however, the question rose whether this agent could eradicate the tumor before death of the patient. In order to find more selective anti-tumor agents, derivatives of sulfur mustard were synthesized and evaluated for their anti-tumor activity. Nitrogen mustard (chart 1.1) was the first

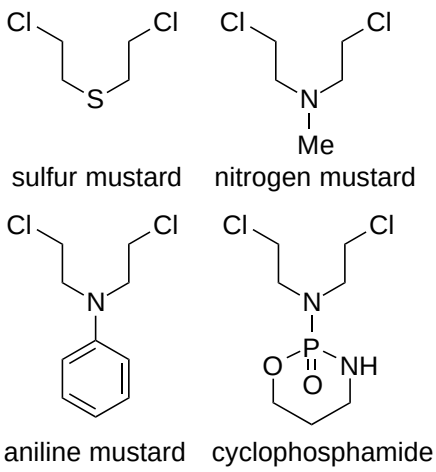


Chart 1.1 *Mustard compounds.*

anti-cancer agent introduced into a clinical trial in 1942 [2]. Analogs of this compound sharing the same mechanism of action (alkylation of DNA) but differing in toxicity and pharmacokinetics appeared soon thereafter (see chart 1.1). The discovery of the anti-tumor activity of these alkylating agents resulted in research for other effective drugs in cancer treatment which led to the development of a wide variety of anti-cancer agents. These drugs are divided into classes based on their mechanism of action or on their origin [3]. The most important classes are *i.* the aforementioned alkylating agents (chart 1.1); *ii.* the platinum based anti-cancer drugs (chart 1.2) making DNA intrastrand cross-linkings (for example cisplatin and carboplatin); *iii.* the anti-metabolites (for example 5-fluorouracil and methotrexate, chart 1.2), interfering with DNA synthesis; *iv.* the natural products such as the vinca alkaloids vinblastine and vincristine and semisynthetic derivatives of epipodophyllotoxins for example etoposide (chart 1.2) (all affecting tubulin functions), and *v.* the anti-tumor antibiotics (for example mitomycin C chart 1.2 and the anthracyclines, chart 1.3). The more recently discovered drug taxol (chart 1.2) and its semi-synthetic analogs are claimed to possess an unique mechanism of action involving cell microtubules [4].

The anthracyclines

This thesis deals with prodrugs of anthracyclines, in view of this, more detailed information concerning the anti-tumor activity of the most important anthracyclines is given.

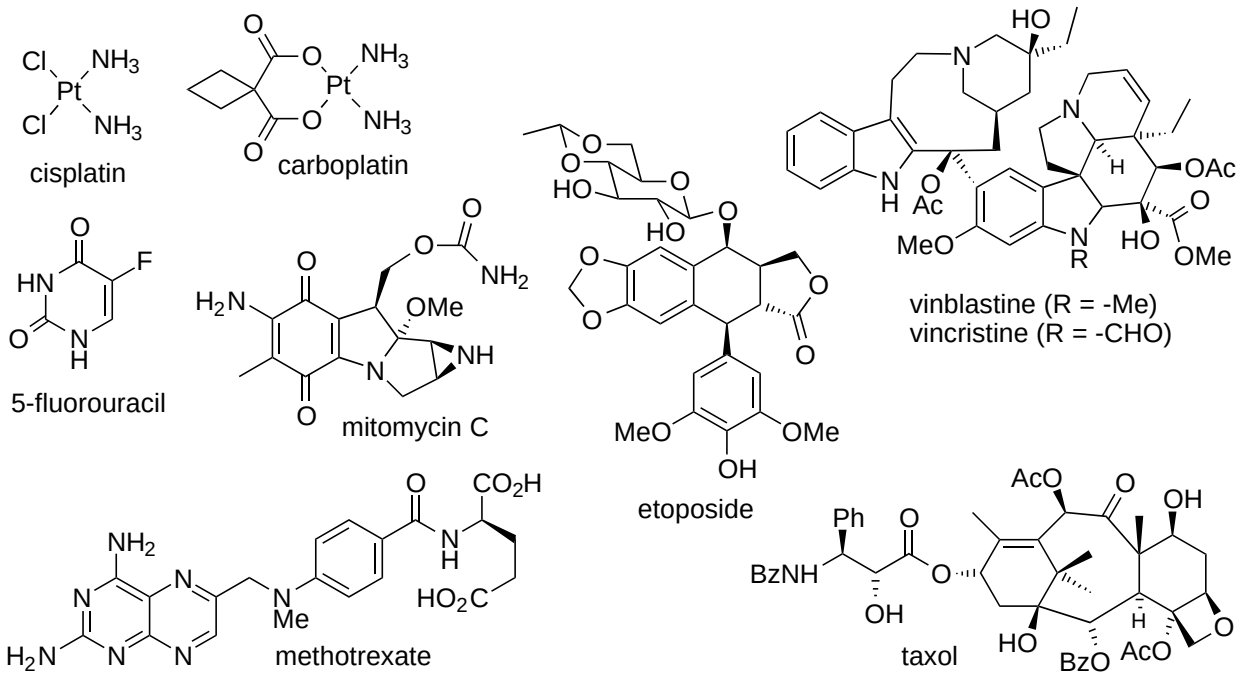


Chart 1.2 *Anti-tumor compounds.*

The anthracycline anti-tumor antibiotics [5] belong to the class of compounds with the widest spectrum of activity in human cancers. The cytotoxic activity of these drugs is ascribed to a number of parallel mechanisms of action. These include *i.* intercalation of the anthracyclinone aglycon moiety between adjacent base pairs in the DNA double

helix causing inhibition of DNA, RNA and protein synthesis; *ii.* stabilization of the topoisomerase II-DNA complex resulting in DNA strand breaks; *iii.* modification of cell membrane functions; *iv.* free radical mediated alkylation of DNA, and *v.* generation of free radicals (e.g. superoxide $O_2^{-}\cdot$) which cause DNA damage. The use of anthracyclines is attended by serious toxic side-effects such as myelosupression, mucositis and cumulative and irreversible cardiotoxicity.

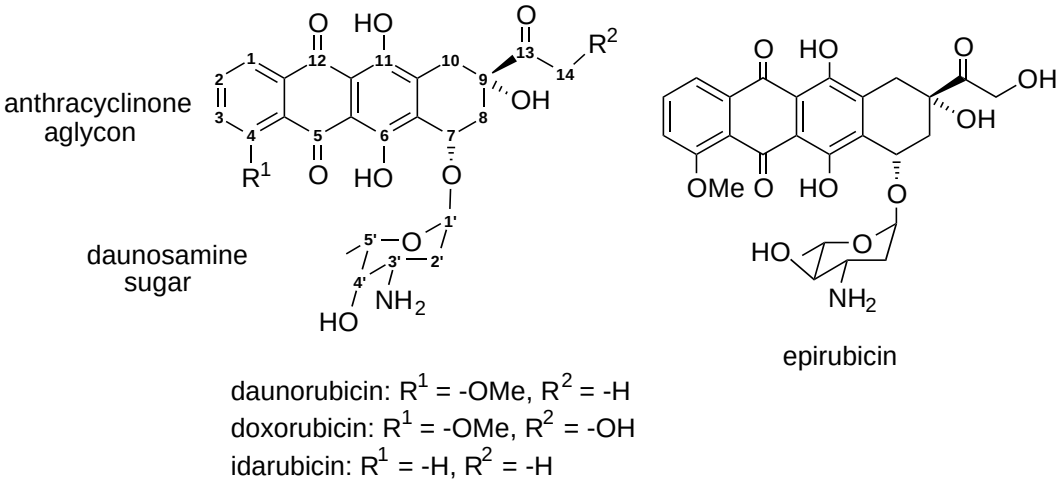


Chart 1.3 *Major anthracyclines.*

The first discovered anthracyclines were isolated from various strains of *streptomyces* and from these, doxorubicin (chart 1.3), discovered in the 1960s, is the most commonly prescribed agent in the treatment of cancer [6]. Daunorubicin, discovered prior to doxorubicin, is almost exclusively used to treat leukaemia because it enters cells very readily. Apart from doxorubicin and daunorubicin two other derivatives (idarubicin and 4'-epirubicin, see chart 1.3) are used worldwide although much less frequently than doxorubicin. Idarubicin can be administered orally (all other anthracyclines are administered intravenously) and is much more active than daunorubicin but it has not replaced doxorubicin and daunorubicin because of its limited bioavailability after oral administration. Epirubicin is more active against different tumors than daunorubicin and doxorubicin and possesses a reduced cardiotoxicity. This compound is rapidly metabolized as a glucuronide [7] and it is becoming used more in the clinic. Major side effects of anthracyclines, however, are myelosuppression and a dose related cumulative and irreversible cardiotoxicity [8] which limits the use of these drugs. An other problem associated with the use of anthracyclines (and other anti-tumor drugs) is the development of multidrug resistance (MDR). In MDR, upon treatment with a single drug, cancer cells acquire resistance to more than one drug by the activation of a cellular excretion system, the P-glycoprotein pump [9]. Resistant cells survive and proliferate leading to a resistant cell clone. Because of the severe side effects of all conventional anti-cancer agents, patients are treated multiple times with a limited dose. This increases the incidence of the developing MDR. When a more selective agent would be employed, a higher dose can be given which diminishes the chance of development of MDR.

Despite efforts of numerous research groups and the synthesis and evaluation of anti-tumor activity of more than 2000 anthracycline analogs, a drug with a higher therapeutic index than doxorubicin has not been developed and doxorubicin remains a major cytostatic agent [10].

1.3 Selective chemotherapy

The insufficient selectivity of conventional cancer chemotherapeutic agents arises from the lack of distinction between tumor and normal cells. Most of the available anti-tumor drugs are specifically toxic to proliferating cells and therefore tumor cells are slightly more sensitive to cytostatics compared with normal cells. Nevertheless therapeutic indices of anti-cancer drugs are too low resulting in unacceptable damage to healthy tissue upon chemo treatment.
A variety of approaches to improve the effectiveness and tumor cell selectivity of anti-cancer agents is under development in an increasing number of research groups. In this context the majority of the research teams utilize antibodies to direct cytotoxicity to tumor tissue. To target cytotoxicity to neoplastic cells, tumor cell-specific antibodies are equipped with for example *i.* drugs [11], *ii.* radioisotopes[12], *iii.* cytotoxins[13], *iv.* liposomes carrying drugs[14], *v.* enzymes[15] or *vi.* genes expressing enzymes[16]. Because of the limited number of antigens present on a tumor cell surface it is often impossible to deliver cytotoxic concentrations of a drug at the tumor site. Apart from this, the number of drug molecules that can be bound to an antibody without loss of antibody binding capacity is limited[17], setting a maximum to the number of drug molecules targeted to the tumor. Alternatively, only a limited dose of an antibody-radioisotope conjugate can be given to a patient because of radiation of the conjugate to normal tissue prior to localization. Because of this, only the most radiosensitive tumors can be treated. In another strategy, when an enzyme which generates toxicity is targeted to a tumor, a much larger effect can be expected because of the catalytic nature of the system. Cytotoxicity generated by an enzyme targeted to a tumor was first demonstrated in 1973[18]. In this study, glucose oxidase was covalently bound to an antibody and generated H_2O_2 from glucose. Disappointingly, very little cytotoxicity was obtained.
A more promising approach is to use a relatively non-toxic prodrug that is selectively activated at the tumor site by a targeted enzyme. This approach was independently introduced by the group of Bagshawe[19] in London and by the group of Senter[20] in Seattle and is denoted as **A**ntibody **D**irected **E**nzyme **P**rodrug **T**herapy, ADEPT. An other approach of directing an enzyme to a tumor in order to activate a prodrug is GDEPT[16] : **G**ene **D**irected **E**nzyme **P**rodrug **T**herapy. In this strategy a gene encoding for an enzyme is targeted to cancer cells. This enzyme is chosen to convert a prodrug to the parent drug. A difference of this method compared to ADEPT is that the enzyme remains located inside the cell and an immune respons of the host is not expected. Therefore, in contrast to ADEPT, a prodrug in GDEPT must be able to penetrate cells in order to be activated by the matching enzyme which is expressed intracellularly. Alternatively, when a retrovirus is used to deliver the gene encoding for an enzyme

which is able to convert a prodrug to the parent drug, the concept is called **V**irus **D**irected **E**nzyme **P**rodrug **T**herapy, VDEPT[21].

In this thesis the synthesis and evaluation of prodrugs of anthracyclines designed for use in ADEPT is described. The ADEPT concept has been extensively reviewed in the last few years[22] . Therefore only a concise summary of it will be given in this chapter, especially focusing on the prodrugs used in ADEPT.

1.4 Antibody Directed Enzyme Prodrug Therapy (ADEPT)

1.4.1 General

The ADEPT[23] approach of treating cancer (see figure 1.1) builds on the discovery that tumors have specific antigens on their cell surface. In this anti-cancer therapy a monoclonal antibody (mAb) which is specific for tumor antigens, carries an enzyme to the tumor site (step 1). After the mAb-enzyme conjugate has been localized at the tumor site and non-tumor associated conjugate has cleared from plasma and other tissue, a relatively non-toxic prodrug is administered which is converted to the parent cytotoxic drug by the targeted enzyme (step 2).
Because of the catalytic nature of the ADEPT system, a higher local concentration of the drug at the tumor site can be obtained compared to chemotherapy using a mAb-drug conjugate. The lower toxicity of a prodrug used in ADEPT compared to the parent drug itself allows administration of a higher dose of the prodrug compared to conventional chemotherapy. Consequently, higher concentrations of the drug at the tumor site can be achieved.
Although only a limited number of cancer cells express an antigen on their cell surface, this is not expected to be critical for ADEPT. When the active drug is generated by the mAb-enzyme conjugate bound to an antigen positive tumor cell, it can diffuse through the tumor mass and enter a tumor cell which does *not* express the antigen. This is referred to as the bystander effect[24], see figure 1.1. The main problems associated with the ADEPT approach of treating cancer are: *i.* development of host antibodies against the mAb-enzyme conjugate, *ii.* too slow clearance of the non-tumor-associated mAb-enzyme conjugate from the circulation (a number of strategies has been developed to accelerate clearance of the conjugate after tumor localization[25]) and *iii.* cross-reactivity of mAb-enzyme with normal tissue as most tumor antigens are not exclusively tumor-specific. A first clinical experience with an ADEPT system employing a nitrogen mustard prodrug and a mAb-carboxypeptidase G2 conjugate was reported recently by the group of Bagshawe[33c, d] (see section 1.4.3) and gave a strong impetus to the development of the ADEPT approach of treating cancer.
A recent development in the field of ADEPT is the use of humanized catalytic antibodies to activate a specific prodrug. The rationale behind this strategy is that such a humanized catalytic antibody (also called abzyme) is highly specific for a certain substrate and is non-immunogenic. This approach is called ADAPT: **A**ntibody **D**irected **A**bzyme **P**rodrug **T**herapy[26].

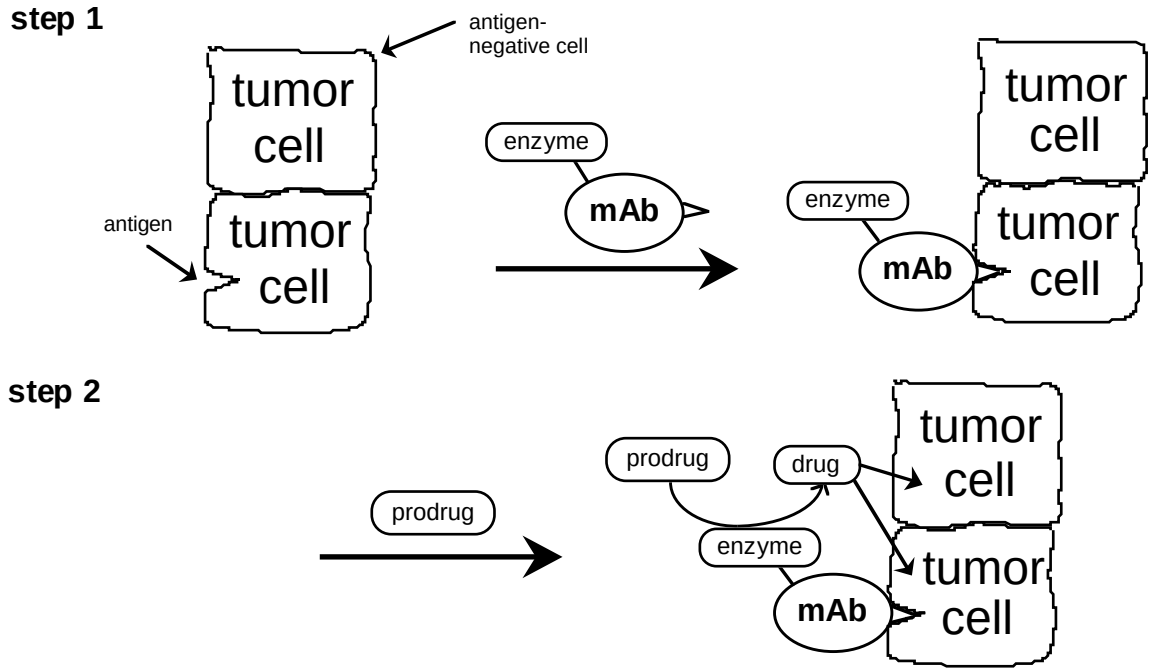


Figure 1.1 *The concept of ADEPT.*

1.4.2 mAb-Enzyme conjugates in ADEPT

Enzyme

A number of prerequisites must be met for the enzyme in the mAb-enzyme conjugate to be optimal for use in ADEPT. Among these are: *i.* optimal pH for enzyme action should be close to that of tumor extracellular fluid, *ii.* preferably no requirement for a cofactor, *iii.* low level of enzyme in plasma and *iv.* high specificity of the enzyme for the prodrug. Most ADEPT systems described in the literature utilize an enzyme of non-human origin. These enzymes were chosen because of the absence of a human homolog resulting in minimal aselective prodrug activation. Therefore, non-human enzymes enable a highly tumor-selectivity ADEPT system. A general disadvantage of the use of such non-human enzymes is the development of host antibodies that restricts the use of the mAb-enzyme conjugate. In the first clinical trial described in the literature [33] the development of host antibodies was anticipated with the administration of an immunosuppressive agent.

Alternatively, the use of an enzyme of human origin does not lead to immunogenicity against the mAb-enzyme conjugate but can lead to aselective prodrug activation by endogenous enzymes. This results in a higher toxicity of the ADEPT system for non-target tissue.

Monoclonal antibody-enzyme conjugate

Important characteristics for a mAb-enzyme conjugate are: *i.* high specificity for a tumor associated antigen, *ii.* no internalization into the tumor cell, *iii.* stability under physiological conditions, *iv.* rapid clearance from the circulation and *v.* penetrability into the tumor mass.

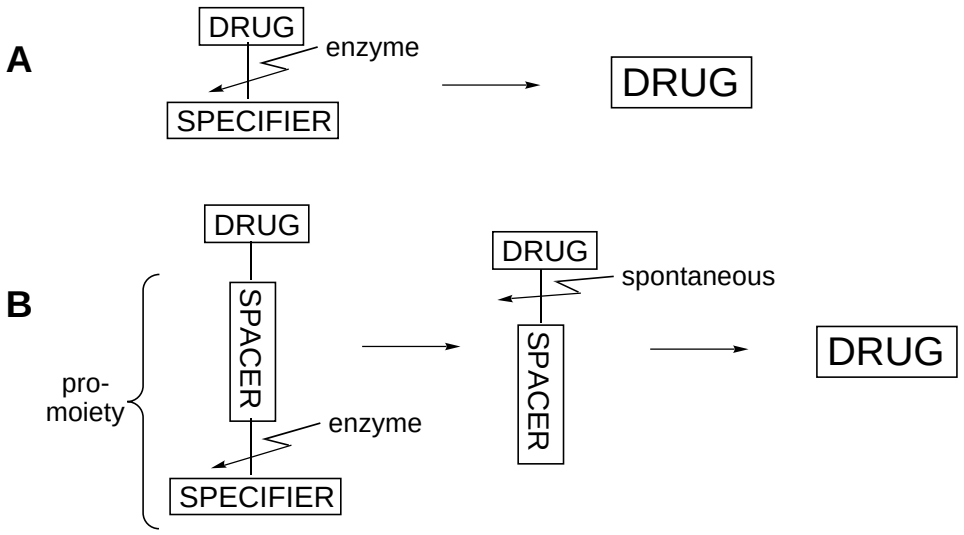
Most of the mAb-enzyme conjugates used in ADEPT have been prepared by a conventional coupling reaction of mAb and enzyme. Alternatively, using DNA

recombinant technology, a DNA fragment encoding for both the mAb and enzyme can be prepared and transcription of this fragment results in a fusion protein possessing both the mAb binding potency and the enzyme activity. Bosslet *et al.* have referred to the use of a fusion protein in ADEPT as FMPA; **F**usion protein **M**ediated **P**rodrug **A**ctivation[27] This technique facilitates the preparation of a mAb-enzyme conjugate to some extent which is required for extensive clinical trials of ADEPT.

1.4.3 Prodrugs in ADEPT

A prodrug in ADEPT must fulfil a number of conditions to be applicable. Important characteristics for a prodrug are: *i.* reduced toxicity compared to the parent drug, *ii.* stability in serum, *iii.* efficient enzymatic activation only by the targeted enzymes, and *iv.* low cellular uptake. In the literature, a large number of prodrugs designed for use in ADEPT are described. The major obstacles, however, for the use of most of the prodrugs in ADEPT are: *i.* too slow activation rate by the matching enzyme[28], *ii.* premature prodrug activation by endogenous enzymes[29] and *iii.* too high toxicity of the prodrug[30].

Initially, prodrugs were reported which were constructed according to alternative A in scheme 1.1. According to this design, a substrate group for a given enzyme is coupled directly to a cytotoxic drug. Upon contact of the prodrug with the matching enzyme, the drug is generated. The group which is a substrate for the enzyme is referred to as the specifier group. A problem encountered in this type of prodrug is a too slow enzymatic activation rate. In most cases, this was attributed to steric hindrance of the prodrug on the enzymatic scission reaction.



Scheme 1.1 *Prodrugs with and without spacer group.*

A possible way to improve the rate of activation of a prodrug is the incorporation of a self-eliminating (immolative) spacer moiety between the drug and the specifier group. In this design (alternative B, scheme 1.1), after enzymatic hydrolysis of the specifier group, elimination of the spacer from the spacer-drug molecule is promoted and the free drug is generated. The spacer plus connected specifier is referred to as the pro-

moiety. The use of such a three-component prodrug system enables optimization of enzymatic activation characteristics merely by structural variation of the spacer. Examples of spacer-containing prodrugs are given in scheme 1.3.
The first example of an ADEPT system was described by Bagshawe and co-workers in 1987[31]. In this system a mAb-carboxypeptidase G2 conjugate was used to activate prodrug **1** (chart 1.4) releasing a nitrogen mustard drug and glutamic acid. Originally, the mAb-enzyme conjugate was intended to be used without prodrug because it was believed to kill cancer cells *via* folate deprivation[32].The combination of prodrug **1** and the mAb-carboxypeptidase G2 conjugate is the first ADEPT system that advanced into human clinical trials[33] . The group of Bagshawe and Springer has devoted a great deal of research to the application[34] and optimization[35] of prodrug **1** in ADEPT. A major limitation of this anti-cancer therapy, however, is the development of antibodies against the bacterial carboxypeptidase G2 enzyme so that immunosuppression using cyclosporin was necessary.

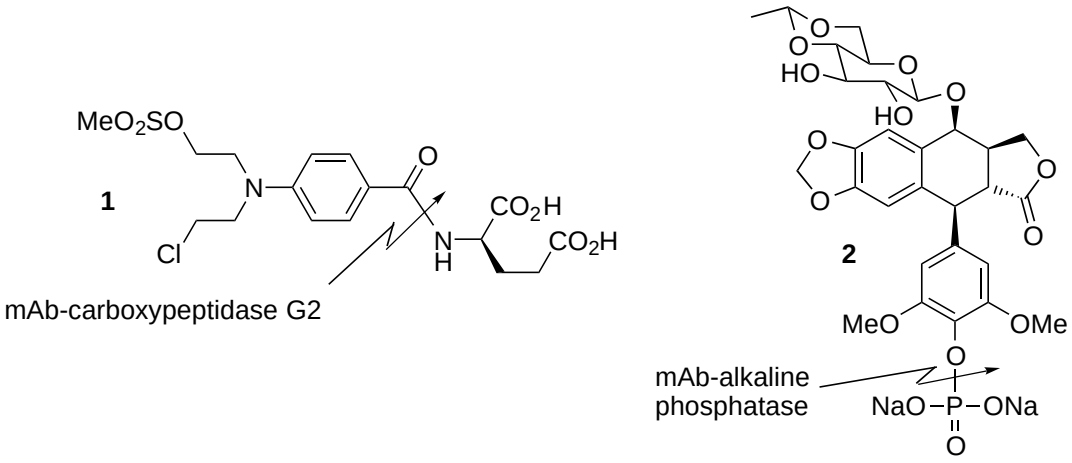


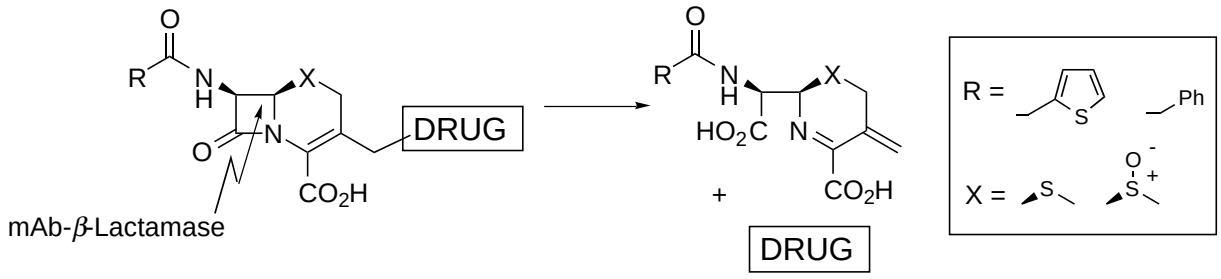
Chart 1.4 *Early ADEPT prodrugs.*

Other early reports[36] by Senter and co-workers at Bristol-Myers Squibb on an ADEPT system describe the use of a mAb-alkaline phosphatase (AP) conjugate. In this system, initially etoposide phosphate **2**[37] (chart 1.4) was used as a prodrug. Other phosphates of anti-cancer drugs studied as prodrug in combination with a mAb-AP conjugate include mitomycin C[38], hydroxyaniline mustard[39], daunorubicin[40], doxorubicin[36c] and taxol (with [41] and without[42] spacer). A serious restriction of the use of AP activated prodrugs, however, is the abundance of phosphatase enzymes in human serum and tissue, resulting in non-specific prodrug activation.

Because comprehensive reviews treating prodrugs which are developed in the context of the ADEPT concept have emerged in the literature[22] (especially refs. 22b and 22g), only some representative examples of prodrug/mAb-enzyme system will be given in this section.

β-Lactamase activated prodrugs

β-Lactamase enzymes are a defence mechanism of bacteria against *β*-lactam antibiotics. These enzymes are readily available and highly selective, and have no human homolog. To prepare *β*-lactamase activated prodrugs, a wide variety of anti-cancer agents have been coupled to the cephalosporin group and are activated according to scheme 1.2.



Scheme 1.2 *Prodrugs activated by β-lactamase.*

Drugs used in this context include methotrexate[43], 5-fluorouracil[43], vinblastine analogs[44], doxorubicin[45], taxol (with spacer)[46], nitrogen mustard drugs[47], mitomycin C[48] and carboplatin analogs[49] (for the structures of these drugs, see section 1.2).

Penicillin V and G Amidase

Penicillin V amidase is a bacterial enzyme selective for *p*-hydroxyphenoxy acetyl containing substrates whereas penicillin G amidase hydrolyses phenylacetamides (chart 1.5). Penicillin V amidase activated prodrugs[50] were prepared from doxorubicin and melphalan (a nitrogen mustard derivative). Penicillin G amidase activated prodrugs[51] were prepared from doxorubicin, melphalan and palytoxin[52] (one of the most potent cytotoxins known).

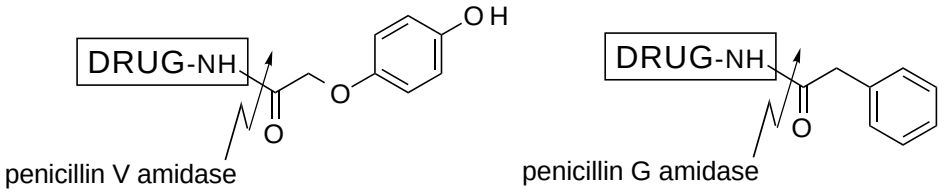


Chart 1.5 *Penicillin amidase based prodrugs.*

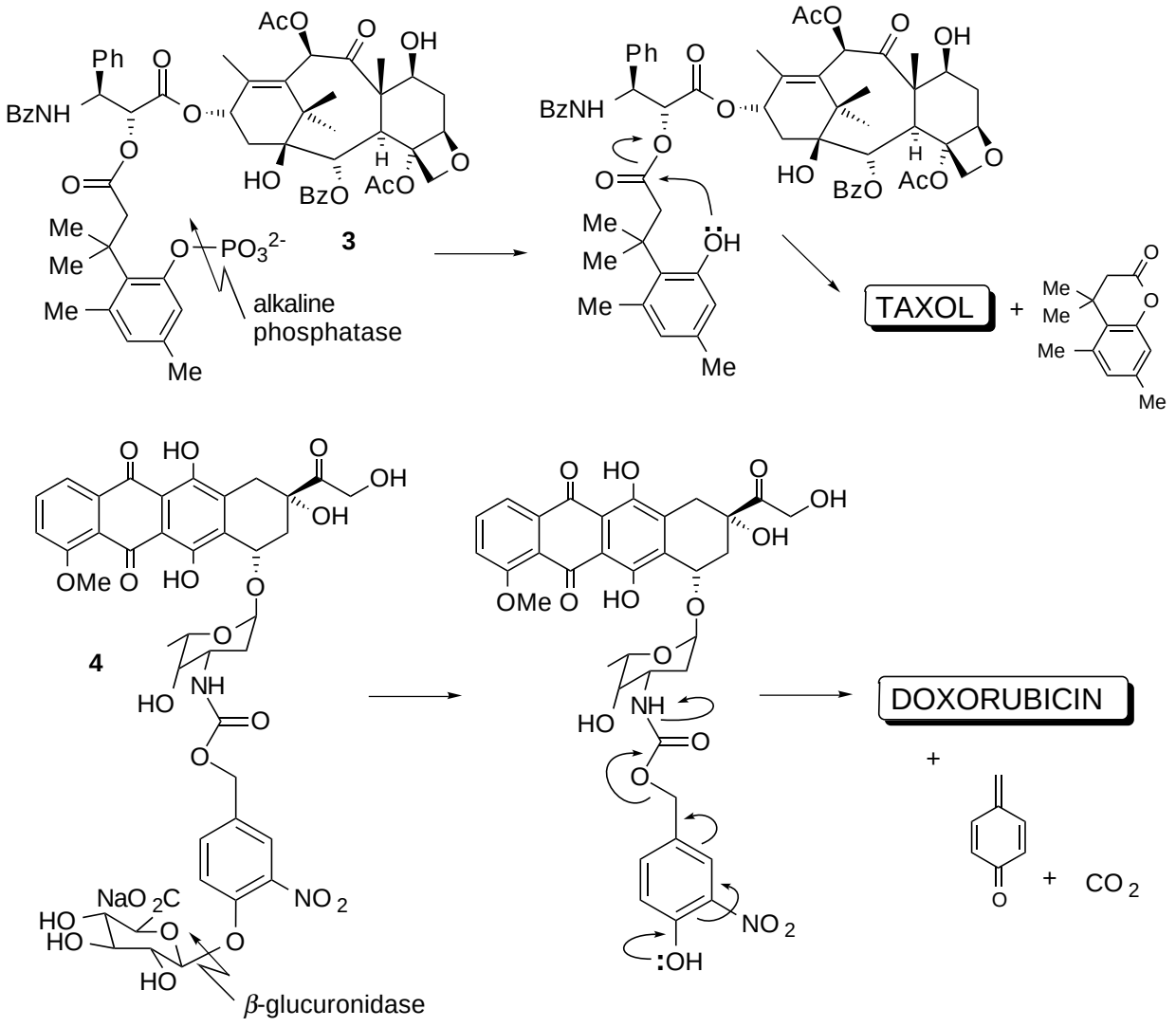
Other enzymes frequently used in the context of ADEPT include *i.* nitroreductase, *ii.* carboxypeptidase A and B, and *iii.* cytosine deaminase.

Glycosidases

β-Glucuronidase and *α*-galactosidase are human intracellular enzymes present in the circulation in only minor concentrations. These enzymes were chosen for the use in ADEPT because their low immunogeneticity and because *β*-glucuronide and *α*-galactoside derivatives of drugs are much more polar than the drug molecule themselves. Because of this property such glycosyl based prodrugs can hardly enter

cells and hence will have a reduced cytotoxicity. In a recent paper the role of human β-glucuronidase in drug targeting has been reviewed[53] A variety of β-glucuronide containing prodrugs have been prepared from drugs. These include *p*-hydroxyaniline mustard (with [54] and without[55] spacer) and epirubicin[56]. Spaced[57] and non-spaced[58] β-glucuronide and α-galactoside based prodrugs have been prepared from both daunorubicin and doxorubicin. In the literature, these anthracycline prodrugs have been extensively evaluated[59] for their application in ADEPT.
Observations made by Connors *et al.* already in 1966[60] pointed out that certain tumor cells contain high levels of β-glucuronidase. In a more recent paper of Bosslet *et al.*[61] it has been demonstrated that β-glucuronidase is liberated especially from necrotic tumor areas. It was suggested in both articles that this endogenous β-glucuronidase can activate a β-glucuronide containing prodrug. This approach is referred to as mono-therapy. For a more detailed background, see section 6.4.3.

Spacer containing prodrugs



Scheme 1.3 *Spacer containing prodrugs.*

As was mentioned earlier, a possible way to improve the rate of activation of a prodrug is the incorporation of an immolative spacer group between the drug and the specifier group (see alternative B, scheme 1.1). After enzymatic hydrolysis of the specifier group, elimination of the spacer from the spacer-drug molecule is promoted and the free drug is generated. To date, most prodrugs designed for use in ADEPT contain a spacer moiety between specifier and drug to facilitate activation. Examples are given in scheme 1.3. The majority of spacer types used for prodrug preparation eliminate by *i.* intramolecular cyclization (e.g. taxol prodrug **3**[41]) or *ii.* 1,6-elimination (e.g. doxorubicin prodrug **4**[59]).

1.5 Aim of research and outline of this thesis

Upto now a number of ADEPT systems has been developed and described in the literature which all had some drawbacks. Among these are immunogenicity of the mAb-enzyme conjugate, a low prodrug activation rate and a too high prodrug toxicity. The aim of the research presented in this thesis is the design and chemical synthesis of prodrugs of anthracycline anti-tumor agents which are optimal for use in the ADEPT approach of treating cancer.
In chapter 1, the background of cancer chemotherapy is described and a concise overview of selective chemotherapy according to the **A**ntibody **D**irected **E**nzyme **P**rodrug **T**herapy (ADEPT) approach is given.
Chapter 2 describes preliminary research with the objective to select the most advantageous ADEPT system. Prodrugs of daunorubicin were prepared containing a phosphate, sulfate, *β*-glucuronide, *β*-glucoside or *β*-galactoside specifier group. From these the *β*-glucuronide containing prodrug/human *β*-glucuronidase combination was found the most promising system for use in ADEPT.
In chapter 3, *β*-glucuronide-based prodrugs of daunorubicin containing a spacer group between drug and *β*-glucuronyl carbamate specifier in order to facilitate prodrug activation are outlined. The spacer group in these prodrugs was designed to eliminate by an intramolecular cyclization reaction after hydrolysis of the *β*-glucuronyl group by *β*-glucuronidase.
In chapter 4, *β*-glucuronide-based prodrugs of anthracyclines containing a spacer designed to eliminate by an 1,6-elimination process are discribed. The spacer group of these prodrugs was substituted with a chlorine, bromine or alkyl group in order to affect prodrug pharmacokinetics. In the final sections of chapters 3 and 4, an evaluation of the synthesized prodrugs for use in ADEPT is presented.
In chapter 5, new synthetic methods for the stereoselective preparation of *β*-glucuronyl carbamates is described. These tools were developed for the synthesis of *β*-glucuronyl carbamate containing prodrugs.
In Chapter 6, an overview is given of the results obtained in both ADEPT and monotherapy experiments using the most promising prodrug which was developed in the course of the research described in this thesis.

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2 The Selection of a Polar Specifier / Enzyme Combination For Use in ADEPT [1]

2.1 Introduction

Ideal prodrugs are pharmacologically inactive substances which are transformed into active drugs exclusively on the location were they actually exert their effect. This implies that the prodrug should not be activated by the same or other mechanisms at distant sites, leading to undesired side-effects. An additional prerequisite for making the use of a prodrug advantageous in comparison with the the parent drug, is an easy clearance from the bloodstream of a patient so that non-specific chemical or enzymatic prodrug activation is minimized. The inactivation of a cytotoxic drug exerting its action within the cell can be achieved by coupling it to a polar moiety, resulting in a poor cell-membrane penetration of the thus obtained polar drug derivative. As a consequence the derivative will exhibit a reduced cytotoxicity. Moreover, when the polar group is attached to the drug *via* a functional group which is important for the antitumor activity, the cytotoxicity of the prodrug will be reduced further. Activation of these relatively non-toxic prodrugs at the tumor site can be effected by targeted enzymes according to the ADEPT principle (**A**ntibody **D**irected **P**rodrug **T**herapy, see Ch. 1).
A priori the enzyme of choice for an ADEPT system is of human origin to prevent the development of host antibodies to the monoclonal antibody-enzyme (mAb-enzyme) conjugate. Furthermore, plasma levels of the candidate enzyme must be low precluding non-specific prodrug activation. From the prodrug characteristics described in the literature and reviewed in chapter 1, rates of enzyme-mediated activation of a prodrug into a drug as well as prodrug toxicities are critical parameters for an effective application of ADEPT to cancer treatment. To enable the selection of the most promising ADEPT-system, polar prodrugs from daunorubicin, employing phosphate-, sulfate-, glucuronide-, glucoside- and galactoside-specifiers were synthesized and subjected to a series of cytotoxicity- and enzyme hydrolysis assays. Hydrolysis rates were studied using alkaline phosphatase, aryl sulfatase, β-glucuronidase and β-galactosidase [44].

2.2 Target compounds

2.2.1 Phosphates and sulfates

Early ADEPT systems described in the literature involved drug-phosphates that were cleaved by mAb-alkaline phosphatase conjugates (mAb-AP). The first phosphorylated drug was etoposide phosphate **1** [2, 3] (chart 2.1). Others include phenol mustard phosphate **2** [4] and doxorubicin phosphate **3** [5]. Etoposide phosphate was much less

toxic compared to etoposide and was readily converted by mAb-AP. Significant levels of antitumor activity have been obtained *in vitro* and *in vivo* using **1** in combination with a mAb-AP and this provided the first evidence that the combination of a prodrug derived from a clinically approved anticancer drug and a mAb-enzyme conjugate is able to kill antigen-positive tumor cells *in vivo*. Phosphorylated mustard drug **2** displayed similar effects, whereas no further evaluation of doxorubicin phosphate **3** was published, except its rapid conversion into free doxorubicin. A disadvantage of using phosphorylated prodrugs is premature prodrug activation by endogenous phosphatases ubiquitous in biological systems. In 1994 [6], the NDA filed etoposide phosphate **1** which is a water-soluble clinically active prodrug of etoposide. This phosphate **1** is activated by phosphatases present in serum without pretreating a patient with a mAb-AP. Using **1** instead of etoposide, no enhanced tumor-selectivity was found but solubility problems were solved. To study the enzymatic activation of daunorubicin phosphates as a function of the position of the phosphate group, daunorubicin-phosphates **DAU-P1** and **DAU-P2** (Chart 2.2) were designed and prepared.

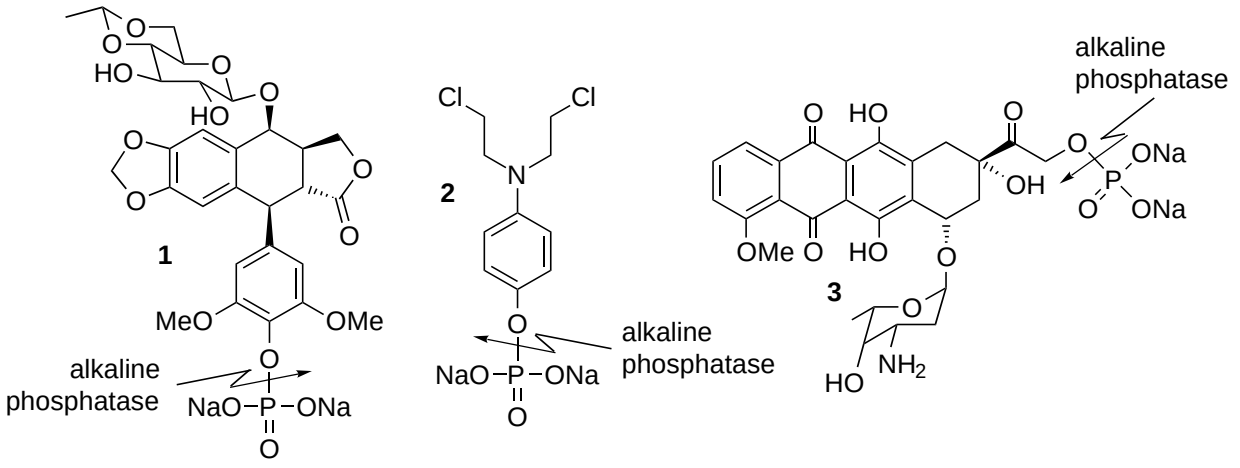


Chart 2.1 *Drug-phosphates described in the literature.*

Sulfatation is a well-known detoxification mechanism in many organisms, thus, anticancer drug-sulfates are conceivable candidates for prodrugs in ADEPT. The exclusive example in this context, described in the literature, is etoposide sulfate (sulfate analog of **1**). This compound could not be hydrolyzed by a variety of arylsulfatases until a Japanese group reported the isolation of an arylsulfatase from soil samples which was indeed capable of hydrolyzing etoposide sulfate [7]. apparently, no further research has been devoted to etoposide sulfate as a prodrug in ADEPT. In this study, daunorubicin sulfates **DAU-S1** and **DAU-S2** (chart 2.2) were synthesized to investigate their potential in selective chemotherapy when used in combination with mAb-aryl sulfatase conjugates.

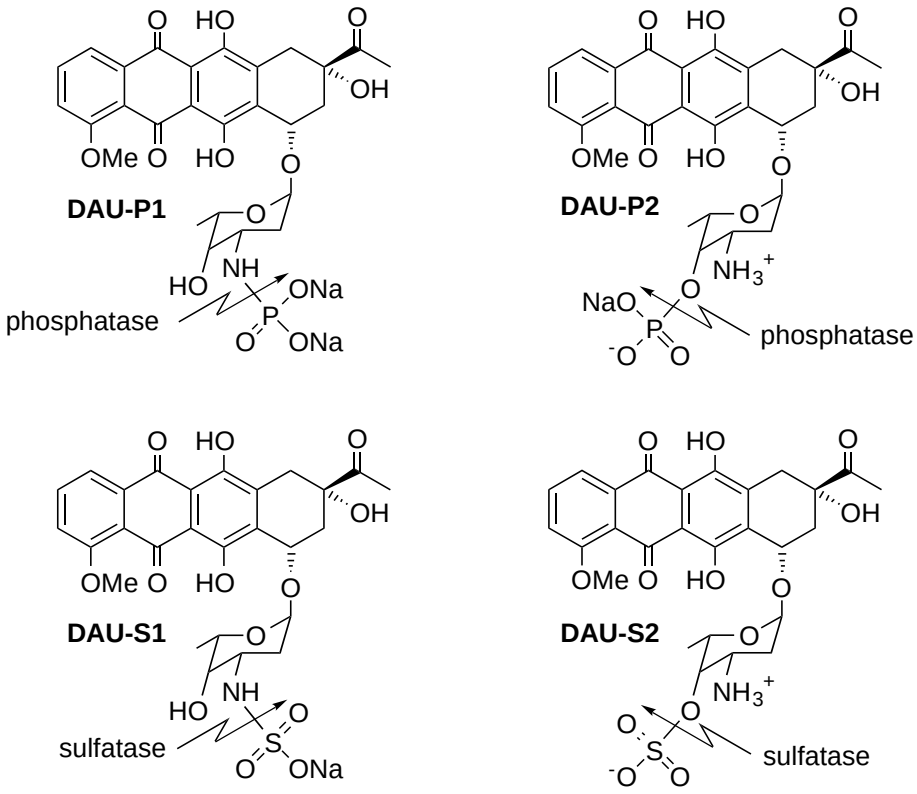


Chart 2.2 *Target compounds; daunorubicin phosphates and sulfates.*

2.2.2 *β*-D-Glucuronides, *β*-D-glucosides and *β*-D-galactosides

β-Glucuronidase, *β*-glucosidase and *β*-galactosidase are human intracellular enzymes present in the circulation in only minor concentrations [8]. As for sulfates, *β*-D-glucuronides are formed in the body in the context of a detoxification process and therefore a drug-*β*-D-glucuronide seems a likely candidate to function as a prodrug in ADEPT. In the literature the synthesis and use in ADEPT of several drug-*β*-D-glucuronides have been described. Among these are the prodrugs of anthracyclines epirubicin 4'-*O*-*β*-D-glucuronide **4** [9] (chart 2.3) and doxorubicin 14-*O*-*β*-D-glucuronide **5** [10]. At clinically achievable concentrations of both enzyme and prodrug at pH = 6.8, both epirubicin glucuronide **4** and doxorubicin glucuronide **5** are activated very slowly by human *β*-glucuronidase [11].

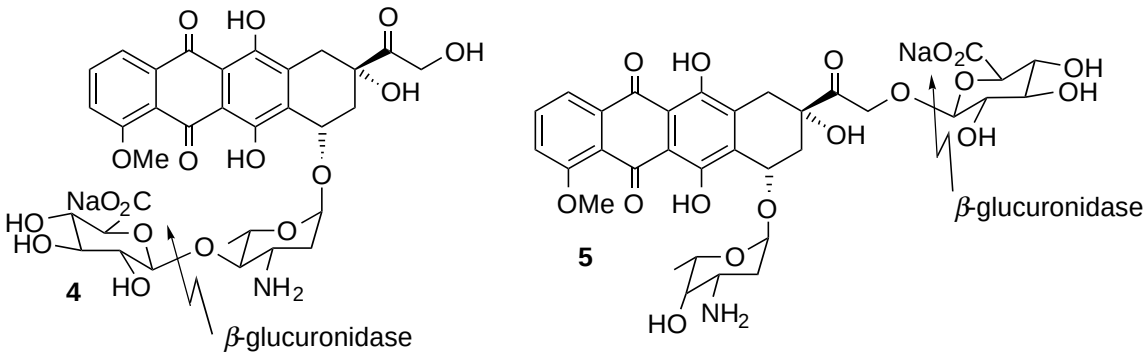


Chart 2.3 *β-D-Glucuronide containing prodrugs.*

Preliminary studies showed that *O*-*β*-D-glucuronyl carbamates **7** (chart 2.4) are fairly good substrates for *β*-glucuronidase. Enzymatic hydrolysis of *O*-*β*-D-glucuronyl carbamates has not emerged in the literature. It was found that model compound **8** (chart 2.4) is readily hydrolyzed by *β*-glucuronidase to give the unstable *N*-phenyl carbamic acid (**9**) which instantaneously decomposes to CO_2 and aniline. The hydrolysis rate of **8** is comparable to that of 4-nitrophenyl-*O*-*β*-D-glucuronide (**6**, R = *-p*-nitrophenol) which is a very good substrate for *β*-glucuronidase.

From this comparison it appears that O-β-D-glucuronyl carbamates ***7*** *are better substrates for β-glucuronidase than alkyl- and aryl-O-β-D-glucuronides* ***6****. In this perspective, it is conceivable that a β-D-glucuronyl carbamate group can be used as a specifier in prodrugs of anthracyclines when coupled to the 3′-amino group of the anthracycline.*

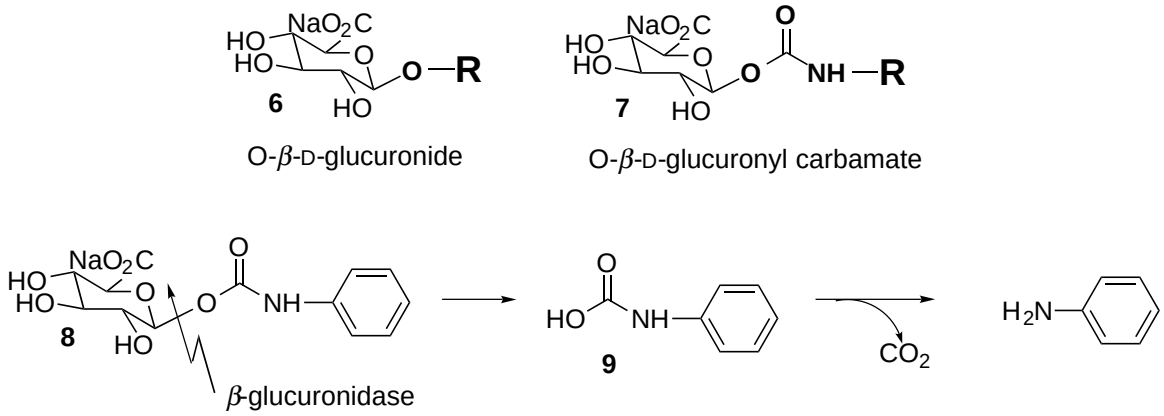
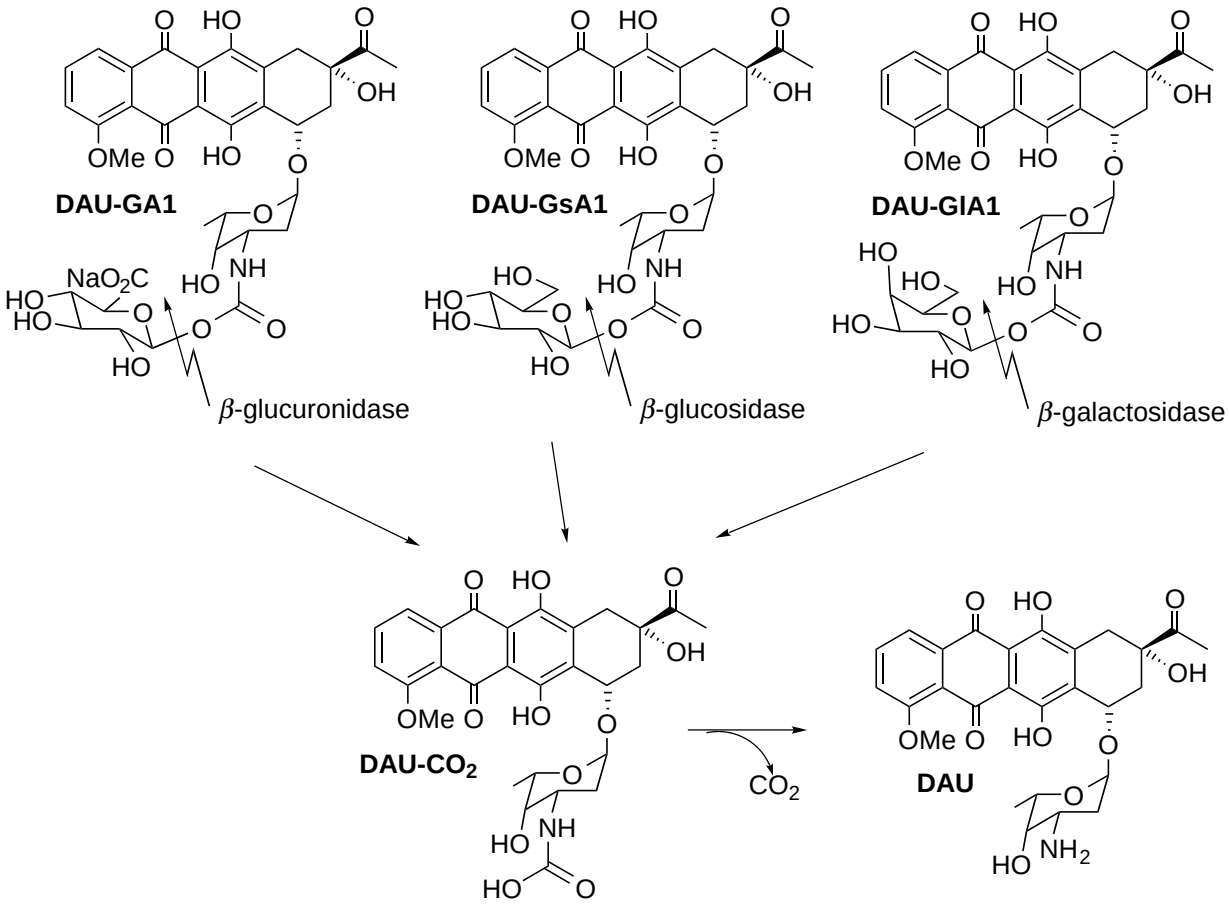


Chart 2.4 *Hydrolysis of O-β-D-glucuronyl carbamates.*

The prodrugs that are designed on this basis are presented in chart 2.5. The polar glucuronyl group is attached to the drug *via* the 3'-amino group which is important for the anthracycline antitumor activity and accordingly the cytotoxicity of the prodrug will be minimized. In addition to *N*-3'-daunorubicinyl *O*-*β*-D-glucuronyl carbamate **DAU-GA1**, the corresponding -*O*-*β*-D-glucosyl and -*O*-*β*-D-galactosyl analogs **DAU-GsA1** and **DAU-GlA1**, respectively, were designed and synthesized and their application in ADEPT was investigated. These novel glycosylated prodrugs are designed to be activated as indicated in chart 2.5. All glycosylated prodrugs in this thesis are named using the nomenclature system shown in chart 2.5.



nomenclature of glycosylated prodrugs:

anthracycline: **DAU** = daunorubicin
DOX = doxorubicin (Ch. 4)
IDA = idarubicin (Ch. 4)

specifier: **G** = β–D-glucuronyl carbamate
Gs = β–D-glucosyl carbamate
Gl = β–D-galactosyl carbamate

specifier, drug, spacer type, spacer number

...-.. .. .. (for example "**DAU-GsA1**")

spacer type: **A** = -CO_2 spacer (Ch. 1), or spacer expulsion by 1,4- or 1,6-elimination (Ch. 4)
B = spacer expulsion by cyclization (Ch. 3)

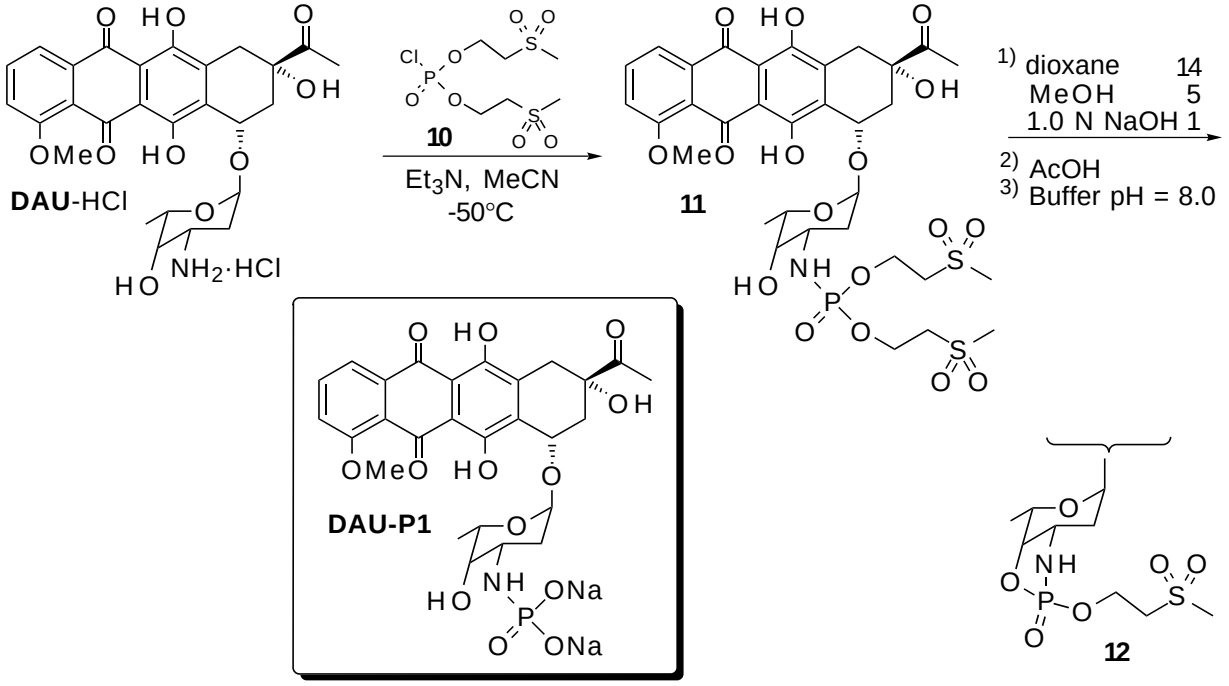
spacer number: numbering of prodrugs of same type

Chart 2.5 *Target compounds; N-3′-daunorubicinyl O-β-glycosyl carbamates and activation pathway to free drug.*

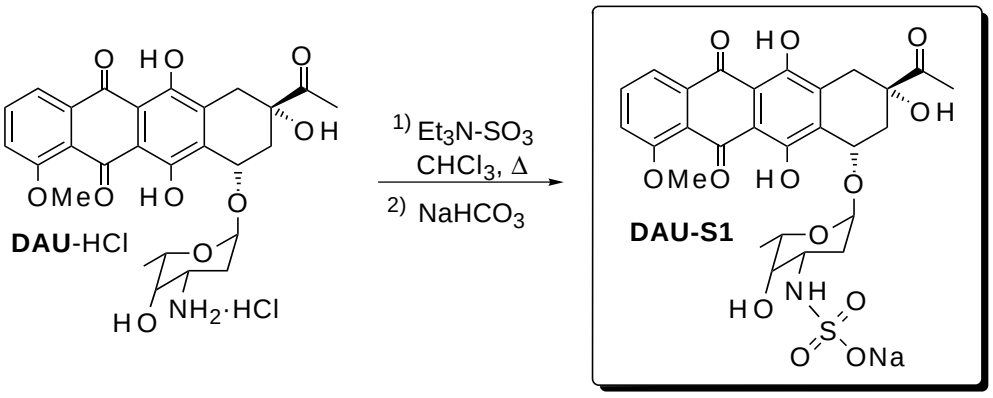
2.3 Synthesis of daunorubicin-*N*-phosphate and -sulfate

The synthesis of daunorubicin-*N*-phosphate and -*N*-sulfate was readily accomplished. Making use of the much larger nucleophilicity of the 3'-amino group of daunorubicin, protection of the hydroxyl group in the molecule was not necessary. Daunorubicin-*N*-phosphate **DAU-P1** (scheme 2.1) was obtained in 40% overall yield after reaction of daunorubicin hydrochloride **DAU**-HCl with the *bis*-[2-(methylsulfonyl)ethyl]-protected (MSE) phosphochloridate **10** [12], immediately followed by deprotection of the

phosphate group of product **11** and purification on a reversed phase C_{18}- column. The cyclic phosphate **12** was isolated as a major side product from the phosphorylation reaction.



Scheme 2.1 *Synthesis of daunorubicin-N-phosphate.*

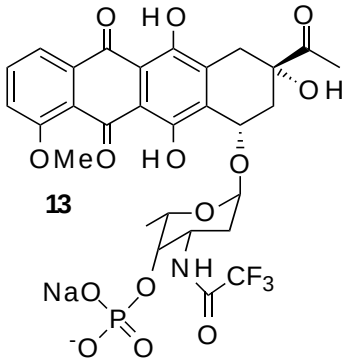


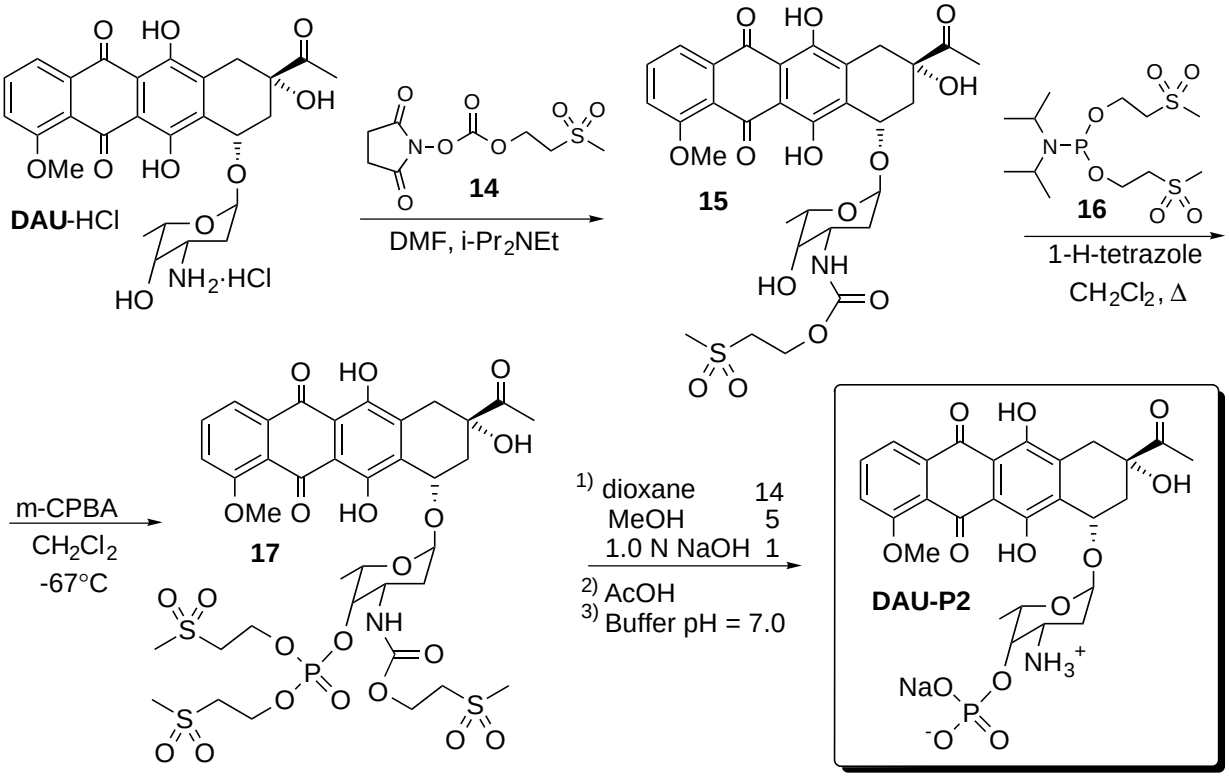
Scheme 2.2 *Synthesis of daunorubicin-N-sulfate.*

Daunorubicin-*N*-sulfate **DAU-S1** was readily obtained in 71% yield by refluxing daunorubicin-HCl with sulfur trioxide-triethylamine complex in chloroform [13], followed by conversion of the resulting triethyl ammonium salt into the sodium salt using sodium bicarbonate (scheme 2.2). The phosphamide **DAU-P1** and sulfamide **DAU-S1** are highly soluble in water and displayed no detectable decomposition in aqueous solution.

2.4 Synthesis of daunorubicin-4'-*O*-phosphate and -sulfate

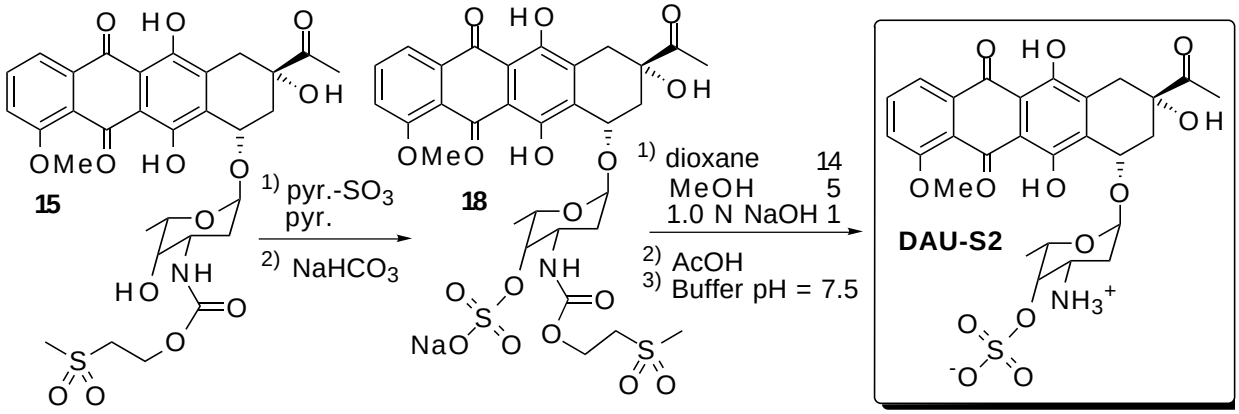
For the preparation of daunorubicin-4'-*O*-phosphate and -sulfate, the 3'-amino group needs to be protected. Leaving the amino group unprotected during phosphorylation and sulfatation, led to **DAU-P1** and **DAU-S1**, respectively (section 2.3). Traditionally, the trifluoroacetyl (TFA) group is used in anthracycline chemistry to protect the 3'-amino group [14]. The TFA group was used in the synthesis of **DAU-P2** but removal of the TFA group from phosphate **13** without hydrolysis of the 4'-*O*-phosphate group was impossible. Therefore, a new protective group strategy is required for the synthesis of the **DAU-P2** and **DAU-S2**. It was suggested that using the -C(O)OMSE moiety to protect the 3'-amino group [15], along with the *bis*-MSE protected phosphorylating agent **10** (scheme 2.1), simultaneous deprotection of both the amino- and phosphate-groups is possible. When applied to the synthesis of **DAU-P2**, protection of the 3'-amino group employing *O*-MSE *N*-succinimidyl carbonate **14** [15] prior to phosphorylation of daunorubicin at the 4'-hydroxyl position, resulted in *N*-3'-daunorubicinyl *O*-MSE-carbamate **15** (scheme 2.3). This protected intermediate **15**, however, appeared to be inert to phosphorylation at the 4'-hydroxyl group using phosphorochloridate **10**.





Scheme 2.3 *Synthesis of daunorubicin-4'-O-phosphate.*

However, phosphitylation employing phosphoroamidite **16** [16] *in situ* followed by *m*-CPBA mediated oxidation of the phosphorus atom, yielded the desired *tris*-MSE-protected intermediate **17**. Simultaneous removal of all three MSE groups afforded daunorubicin-4'-*O*-phosphate **DAU-P2** in 74% overall yield. Because of the extremely fast (<1 min) *β*-eliminative deprotection process [17], no alkaline hydrolysis of the phosphate ester takes place.
Daunorubicin-4'-*O*-sulfate **DAU-S2** was prepared analogously in an overall yield of 51% by sulfatation of the 4'-hydroxyl group of **15** with sulfur trioxide-pyridine complex [18] followed by deprotection of the 3'-amino group of sulfate **18** (scheme 2.4).
Daunorubicin phosphate **DAU-P2** was appreciably soluble in water, whereas the sulfate **DAU-S2** dissolved moderately in water and partly precipitated on standing.



Scheme 2.4 *Synthesis of daunorubicin-4'-O-sulfate.*

2.5 Synthesis of *N*-3'-daunorubicinyl *O*-*β*-D-glycosyl carbamates

2.5.1 Introduction on *O*-*β*-D-glycosyl carbamates

In the proceeding sections of this chapter and in the following chapters, mainly *O*-*β*-D-glucuronyl carbamates and to a minor extent, *O*-*β*-D-glucosides and *O*-*β*-D-galactosides will be encountered (chart 2.6). Glucuronides are glucosides, oxidized at the C(6)-OH position and galactosides differ from glucosides in the stereochemistry at C(4).

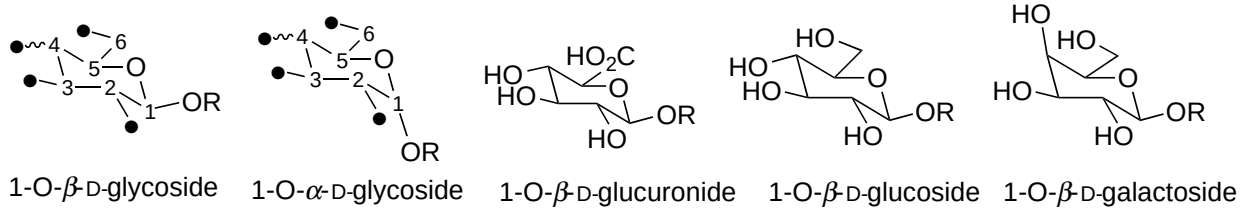
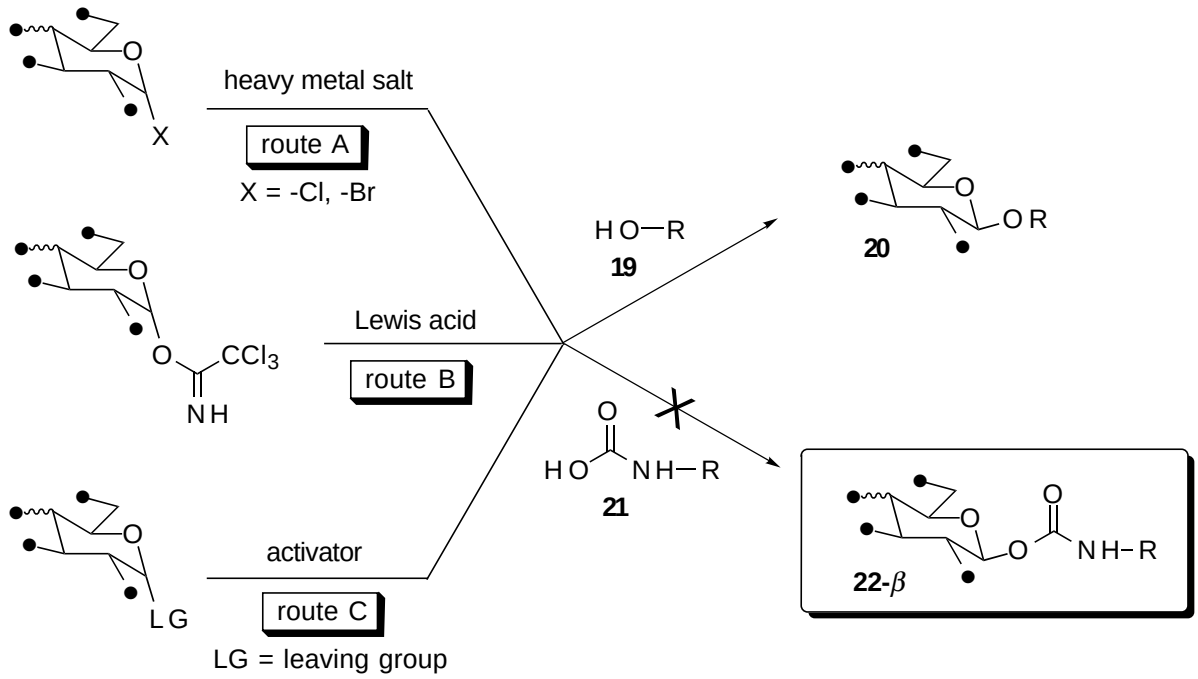


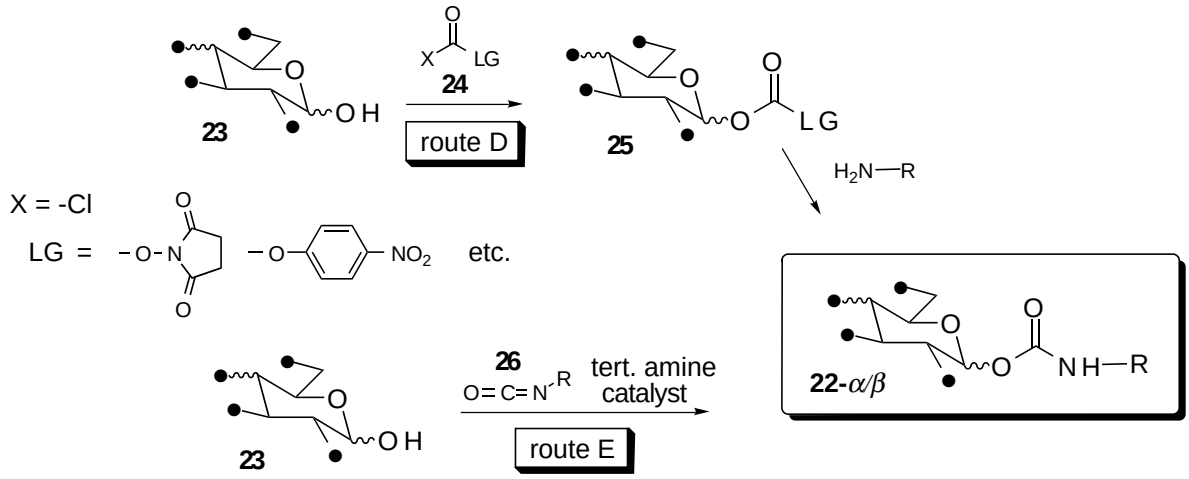
Chart 2.6 *Carbohydrates in this thesis.*

In the last decade, numerous sugar coupling procedures for the synthesis of glycosides **20** have been published. In scheme 2.5, which only represents the *β*-D-glucose- and *β*-D-galactose- series,



Scheme 2.5 *Sugar coupling reactions.*

these coupling reactions are exemplified by the classical Koenigs-Knorr methodology [19] (route A) and by the acetimidate coupling method (route B) more recently developed by Schmidt and co-workers [20]. Several other methods have been described whereby the glycosyl donor is activated prior to reaction with an aglycon alcohol **19** (depicted in general route C). For the synthesis of *O*-*β*-glycosyl carbamates **22**, all these methods cannot be applied because the required carbamic acids **21** are insufficiently stable compounds. Methods for preparing *O*-*β*-D-glycosyl carbamates are very scarce in the literature [21]. New ways for obtaining *O*-*β*-D-glycosyl carbamates are shown in route D and E (scheme 2.6). In a two step procedure (route D), the 1-hydroxyl group of a 2,3,4,6-tetra-protected D-glycosyl donor **23** is first activated as an active carbonate **25** by reaction with chloroformate **24**. In step 2, the active carbonate **25** is condensed with an amine to afford carbamates **22**. The *α*/*β* selectivity of the reaction of anomerically unprotected sugar **23** with **24** is governed by the anomeric effect favoring the formation of the *α*-isomer. The diastereomeric product mixture of **22-***α*/*β* is then separated and the desired *β*-anomer can be obtained as a minor product, if at all.

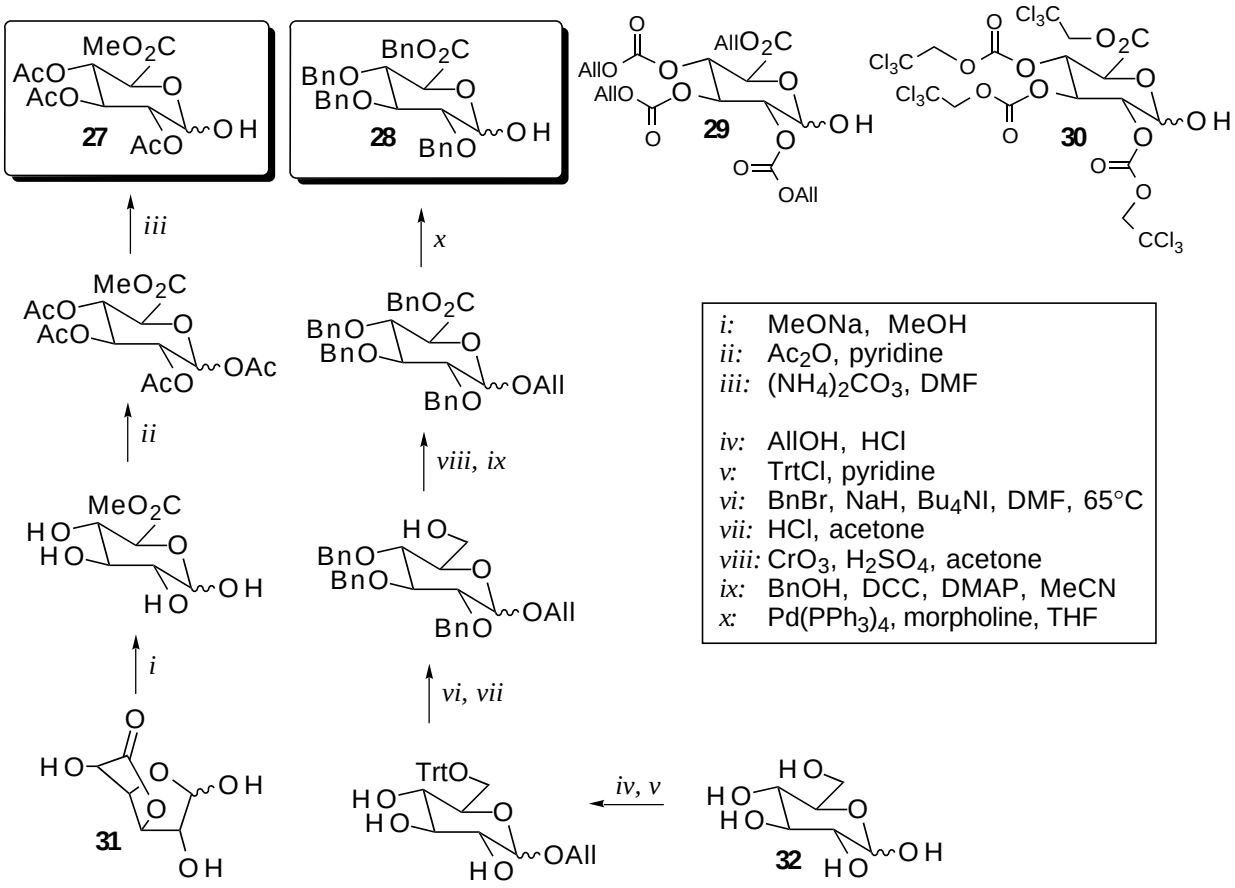


Scheme 2.6 *Synthesis of O-β-D-glycosyl carbamates.*

The synthesis of *O*-*β*-D-glycosyl carbamates by the addition of the 1-hydroxy group of a D-glycosyl donor to an isocyanate (route E), however, is preferred because of its superior *β*-diastereoselectivity. This isocyanate addition reaction generally leads to very high, and in some cases to exclusive *β*-diastereoselectivities. Solvent and catalyst are of profound influence on both the yield and the diastereoselectivity of the reaction. This has been outlined in detail in section 5.1 [22].
Limitations following route E are imposed by substituents in isocyanate **26** because active hydrogen functional groups in -R need to be protected to avoid reaction with the isocyanate group.

2.5.2 Synthesis of *N*-3'-daunorubicinyl *O*-*β*-D-glucuronyl carbamate DAU-GA1

For the synthesis of glucuronides [23] of anthracyclines, an appropriately 2,3,4,6-*tetra*-protected D-glucuronic acid possessing a free 1-hydroxyl group is required. In the literature, methyl 2,3,4-tri-*O*-acetyl D-glucuronate **27** and benzyl 2,3,4-tri-*O*-benzyl D-glucuronate **28** are almost exclusively used for D-glucuronic acid conjugation reactions (scheme 2.7). The allyl-type protected and 2,2,2-trichloroethyl-type protected D-glucuronic acids **29** [24] and **30** [25], respectively, have to be synthesized in nine-step procedures each and therefore, the easier accessible D-glucuronyl donors **27** and **28** are preferred. Compound **27** is readily synthesized from D-(+)-glucurono-3,6-lactone **31** [26] using literature methods [27] for selective hydrolysis of the 1-OAc group while additional acetate groups are retained. It was found that the ammonium carbonate mediated 1-OAc hydrolysis [28] is superior to other methods. Compound **28** is obtained from D-(+)-glucose (**32**) from which the C(6) is oxidized to the carboxylic acid according to literature procedures [29].

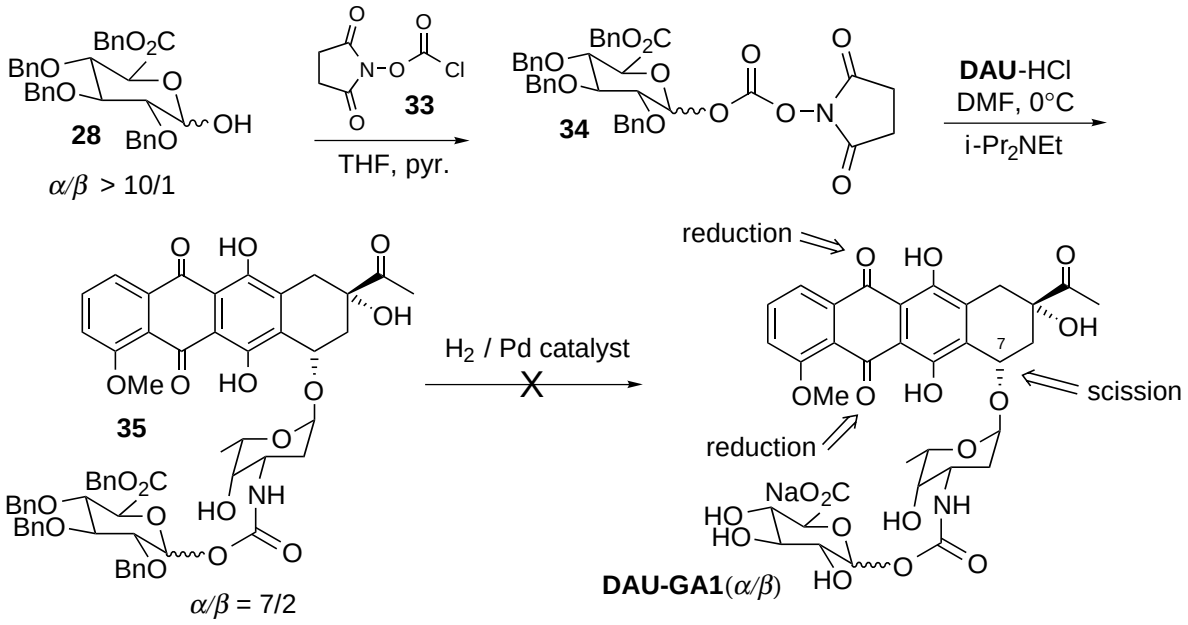


Scheme 2.7 *Synthesis of 2,3,4,6-tetra protected D-glucuronates.*

Benzyl 2,3,4-tri-*O*-benzyl D-glucuronate (**28**, scheme 2.7) was initially used as a D-glucuronyl donor in the synthesis of **DAU-GA1**. Route D (scheme 2.6) was preferred over route E because transformation of the 3'-amino group of daunorubicin to an isocyanate would require protection of the 4'-, 6-, 9- and 11-hydroxyl groups present elsewhere in the molecule. Therefore, **28** was transformed to an active carbonate using *N*-succinimidyl chloroformate **33** [30] which then was further transformed to the tetra-benzyl protected target molecule **35** (scheme 2.8). The diastereoselectivity of the chloroformate addition reaction and the subsequent reaction of the thus obtained active carbonate with daunorubicin is in favor of the α-anomer in accordance with the anomeric effect and resulted in **35**α/β = 7/2. This is similar to the α/β ratio of reactions of **28** with acid chlorides as described by Keglevic and coworkers [31]. The anomeric hydroxyl group of **28** is predominantly in the α-configuration as confirmed by proton NMR; the exact α/β balance could not be ascertained because of overlap of signals of other protons with the 1-H doublet of **28**β. 1-H of **28**α showed a clear doublet with an integral of almost one proton at δ = 5.19 ppm (J = 3.4 Hz) [32]. This observation is opposed to the suggested β-configuration of this compound made by Pravdic and Keglevic in 1965 [29a] deduced from optical rotation data.

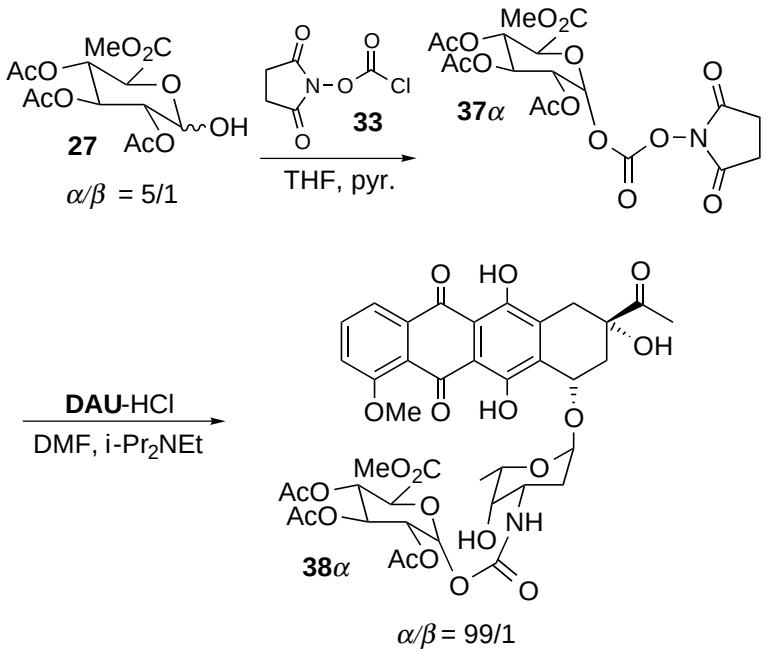
The **35**α/β anomeric pair was separated by circular chromatography and attempts to hydrogenolyze the four benzyl protective groups of the pure β-anomer were undertaken. All attempts to deprotect **35** intended to obtain **DAU-GA1** failed, including classical hydrogenolysis using palladium on charcoal, palladium on Ba_2SO_4, catalytic

transfer hydrogenation using cyclohexene [33] and Pd/C, 1,4-cyclohexadiene [34] and Pd/C, and cyclohexene and $Pd(OH)_2/C$ [35]. In all cases scission of the benzylic ether on C(7) occurred and when a prolonged reaction time was used, reduction of the benzoquinone moiety was observed as well. It is clear that benzyl protection is not compatible with anthracycline chemistry.



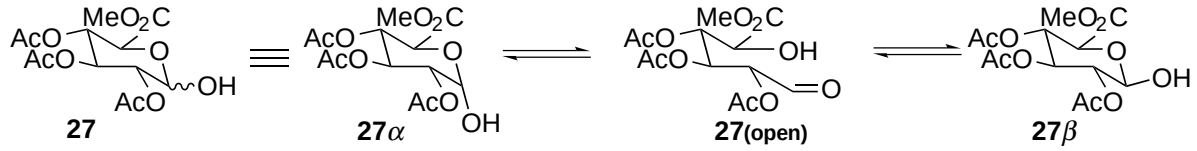
Scheme 2.8 *First attempt to synthesize* ***DAU-GA1****.*

Next methyl 2,3,4-tri-*O*-acetyl D-glucuronate **27** was prepared and used as a D-glucuronyl donor. Analogously to the all-benzyl protected D-glucuronate **28**, compound **27** was activated using **33** and the resulting mixed carbonate **37** was reacted with daunorubicin to furnish **38** (scheme 2.9). Very small amounts of the *β*-anomer were detected. The almost exclusive *α*-diastereoselectivity of the reaction was found to be independent of the solvent used for the reaction. In solution, hemiacetal **27** is an equilibrium between the *α*-and *β*-form of the free anomeric hydroxyl group *via* the open aldehyde **27(open)** as depicted in scheme 2.10. For **27**, the *α/β* ratio in



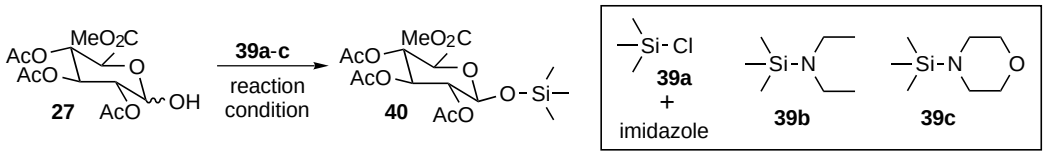
Scheme 2.9 *Second attempt to synthesize* ***DAU-GA1****.*

different solvents is about 5/1, as was determined by proton NMR (see table 5.4 in section 5.1). This is explained by the anomeric effect stating that an electronegative substituent at C(1) of a tetrahydropyranyl ring is preferred in the axial position [36]. When a tertiary amine base was added to a solution of **27** the equilibrium shifted almost completely to the α-anomer, as was determined by proton NMR. On this basis it is understandable that the reaction of **27** with chloroformate **33** catalyzed by pyridine is almost exclusively α-diastereoselective. When the reactions of D-glucuronyl donors **27** and **28** with chloroformate **33** are compared (α/β = 7/2 and 99/1, respectively), **27** results in higher α-selectivities than **28**. This can be attributed to an enhanced anomeric effect in **27**, caused by the presence of the electron-withdrawing acetyl protection groups, in comparison with the more neutral benzyl ether groups in **28**.



Scheme 2.10 *Mutarotation.*

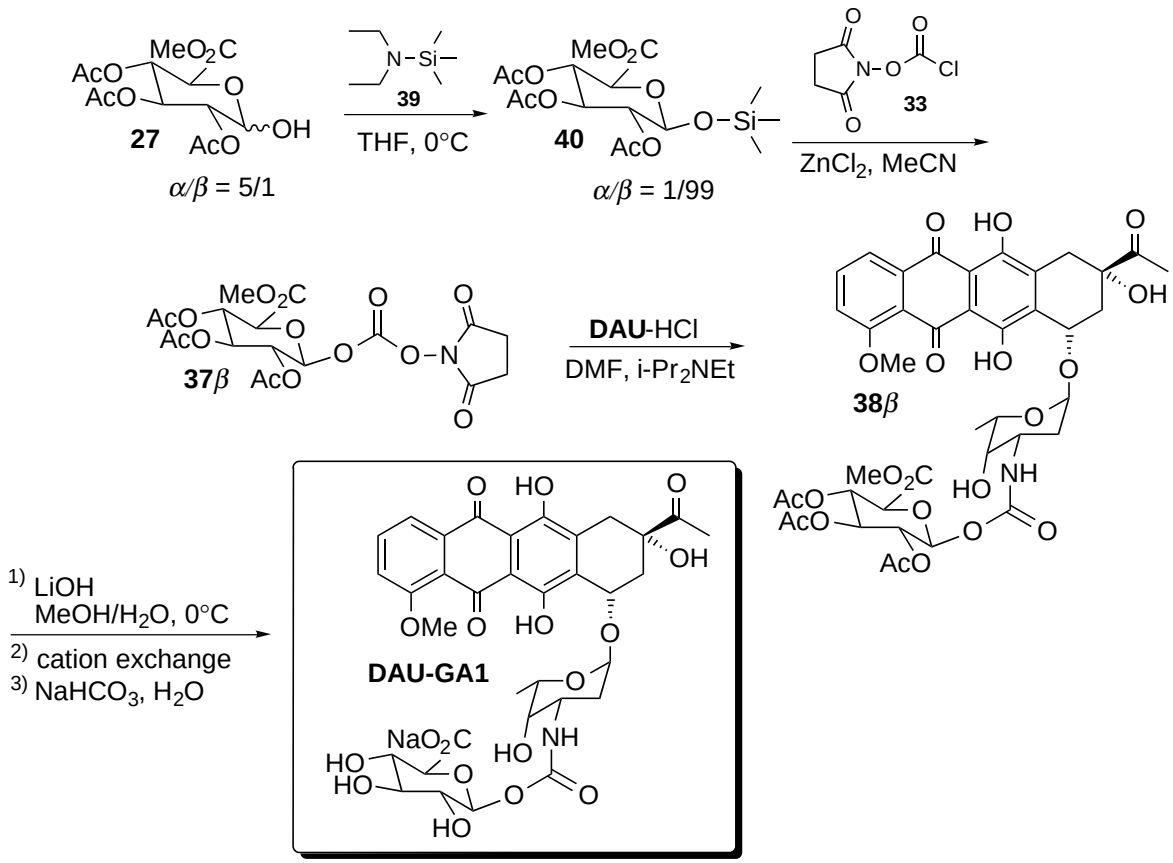
It was argued that the desired β-product could be obtained when **27** ($\alpha/\beta = 5/1$, scheme 2.9) is transformed into the β-OH anomer. A subsequent reaction of the β-anomer of **27** with chloroformate **33** would lead to the β-anomer of **37** (scheme 2.9). A reaction with daunorubicin will then lead to the desired β-product. In this context it is relevant to note that in steroid chemistry it is known that *N*,*N*-diethyl trimethylsilyl amine (**39b**, table 2.1) is able to silylate equatorial hydroxyl groups selectively in the presence of axial hydroxyl functions [37]. Analogously to this, it was reasoned that from the equilibrium of **27**α/β, only the equatorial 1-β-OH group will be silylated. Once the silylated β-anomer is formed, it can be hydrolyzed and the resulting 1-β-OH anomer should be converted to the desired β-product in a rapid reaction to minimize anomerization of the 1-β-OH anomer to the 1-α-OH anomer.



reaction conditions		α/β-ratio of **40** using silylating reagent		
		39a	**39b**	**39c**
$CHCl_3$,	20°C	99/1	53/47	13/87
THF,	20°C	99/1	10/90	15/85
$CHCl_3$,	0°C		9/91	
THF,	0°C		1/99	

Table 2.1 *α/β-Ratio of the silylation reaction of **27** with **39a-c** to **40**.*

To find optimal conditions for the formation of *O*-*β*-trimethylsilyl D-glucuronic acid **40** (see scheme of table 2.1), silylating agents **39a-c** were investigated under a number of different reaction conditions (table 2.1) [38]. Using the least reactive reagent *N,N*-diethyl trimethylsilyl amine (**39b**) in THF at 0°C, highest *β*-diastereoselectivities were obtained. In the mid sixties it was already observed that the 1-*β*-OH anomer of **27** is more nucleophilic than the 1-*α*-OH isomer [39]. Thus, by taking into account the Curtin-Hammett principle, D-glucuronic acid **40** was silylated almost exclusively in the *β*-anomeric form, despite the preponderance of the 1-*α*-OH of **27** in solution.
Following this strategy, *O*-*β*-trimethylsilyl D-glucuronate **40** [40] was converted into the active carbonate 37*β* with zinc chloride [41] and chloroformate **33** (scheme 2.11). Subsequent coupling of **37***β* to daunorubicin yielded the protected prodrug **38***β* in 90% *β*-diastereoselectivity and 38% overall yield. Simultaneous removal of all four protective groups of the D-glucuronide moiety in **38***β* was conveniently achieved in 93% yield in a one step procedure with LiOH in MeOH/H_2O at 0°C furnishing **DAU-GA1**.

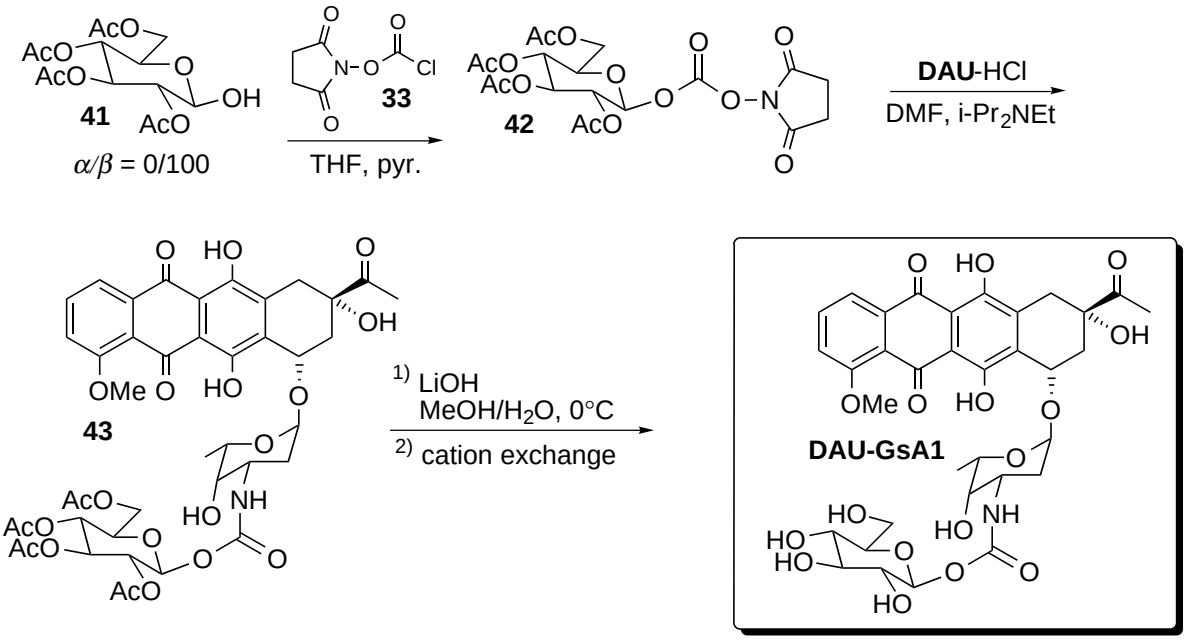


Scheme 2.11 *Synthesis of* ***DAU-GA1****.*

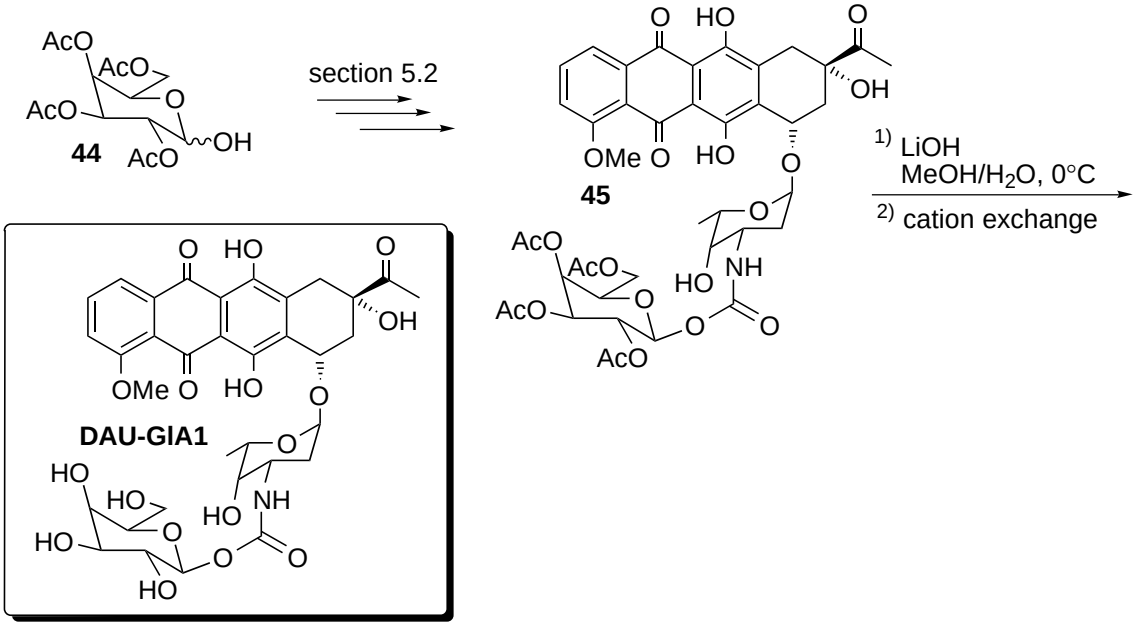
2.5.3 Synthesis of *N*-3'-daunorubicinyl *O*-*β*-D-glucosyl and *O*-*β*-D-galactosyl carbamates DAU-GsA1 and -GlA1, respectively

For the synthesis of the D-glucose- and D-galactose- analogs of **DAU-GA1**, 2,3,4,6-tetra-*O*-acetyl protected D-glucose [42] and D-galactose [43] were used because the acetyl

groups proved to be easily removable without affecting the daunorubicin part of the molecule, see section 2.5.2. *N*-3'-Daunorubicinyl *O*-*β*-D-glucosyl carbamate **DAU-GsA1** (scheme 2.12) was conveniently prepared in 60% overall yield by reaction of the anomerically pure D-glucose tetraacetate-1-*β*-OH **41**, which selectively crystallized from the anomeric mixture of **41***α*/*β*, with chloroformate **33** followed by deprotection of the resulting intermediate **43**. Reaction of the pure anomer **41** with chloroformate **33** is faster than anomerization of the 1-*β*-OH group to an 1-*α*-OH, as only about 10% of the *α*-isomer of **43** was detected by proton NMR.



Scheme 2.12 *Synthesis of* ***DAU-GsA1****.*



Scheme 2.13 *Synthesis of* ***DAU-GlA1****.*

It was not possible to prepare D-galactosyl prodrug **DAU-GlA1** analogously to the D-glucosyl prodrug because 2,3,4,6-tetra-*O*-acetyl D-galactose (**44**α/β) could not be crystallized. The protected D-galactosyl prodrug **45** (scheme 2.13), however, was prepared using a novel glycosyl-carbonyl transfer reaction which is outlined in section 5.2. Deprotection of **45** was readily accomplished with LiOH.

2.6 Evaluation of the synthesized prodrugs for application in ADEPT

2.6.1 Cytotoxicities

Table 2.2 shows a highly decreased cytotoxicity for the sulfate prodrugs, especially the *N*-derivative **DAU-S1** which is 1,600 - 230,000 times less toxic than daunorubicin. On the other hand, the phosphate prodrugs **DAU-P1** and **-P2** exhibit the same or a slightly diminished cytotoxicity on the cell lines compared to daunorubicin. This can be explained by the presence of endogenous phosphatase enzymes in the culture medium. Furthermore, the 4'-*O*-sulfate prodrug **DAU-S2** shows a higher residual activity when compared to the *N*-sulfate analog. This can probably be ascribed to the unoccupied 3'-amino group which is present in the 4'-*O*-sulfate derivative.
The cytotoxicities of the β-D-glucosyl- and β-D-galactosyl-based prodrugs **DAU-GsA1** and **DAU-GlA1**, respectively, (table 2.3) are not as strongly reduced as the β-D-glucuronyl-based prodrug **DAU-GA1** which is at least 2,000 times less toxic than the parent drug. The difference in cytotoxicity between the β-D-glucuronyl prodrug on one hand and β-D-glucosyl and β-D-galactosyl based prodrugs on the other hand, can probably be explained by their large difference in polarity and hence difference in cellular uptake.

	IC50 (μM)					Activation
Compound	A204	MCF-7	T24	WiDr	IgR-37	$t_{1/2}$ (min)
DAU	3	2	2	7	11	
DAU-P1	17	370	2	10	96	n.d.†
DAU-P2	17	89	3	58	45	n.d.†
DAU-S1	4,900	10,400	>180,000	>1,600,000	>1,600,000	∞
DAU-S2	1,300	870	5,500	>1,600,000	3,000	∞

† not determined

Table 2.2 *Cytotoxicities and enzymatic activation rates of phosphate and sulfate prodrugs.*

2.6.2 Enzymatic prodrug activation rates

For the determination of the activation half-lives, the prodrugs were incubated with aryl sulfatase, human β-glucuronidase or bovine liver β-galactosidase [44], respectively, see table 2.2 and 2.3. In the cytotoxicity assays (table 2.2), the phosphate prodrugs were almost as toxic as daunorubicin. It was concluded that these prodrugs undergo premature activation by endogenous enzymes and are of no interest for ADEPT. Therefore, the enzymatic hydrolysis half-lives of the phosphate prodrugs were not

determined. Whereas the novel daunorubicin phosphate prodrugs were hydrolyzed rapidly by endogenous enzymes, the daunorubicin sulfates were not activated to the parent drug by the aryl sulfatase used.

	IC50 (μM)							Activation
Compound	OVCAR-3	MCF-7	WiDr	EVSAT	IGROV	M-19	A-498	$t_{1/2}$ (min)
DAU	0.1	2	7	2	18	2	3	
DAU-GA1	100	11,200	39,500	13,500	36,900	15,800	12,100	8,200 †
DAU-GsA1		125	370	280	520	200	250	>5,000 ‡
DAU-GlA1	6							>5,000 ‡

† human β-glucuronidase, ‡ bovine liver β-galactosidase [44]

Table 2.3 *Cytotoxicities and enzymatic activation rates of β-D-glycosyl prodrugs.*

The β-glycosyl-based prodrugs **DAU-GA1**, **-GsA1** and **-GlA1** (table 2.3) were hydrolyzed at a rate which must be considered too low to be applicable in ADEPT. It was shown in the literature [11a, 45] that prodrugs having an enzymatic hydrolysis rate comparable to those of the aforementioned three β-glycosyl prodrugs are less suitable to be used in ADEPT.

These results indicate that prodrugs consisting of a β-D-glucuronyl-carbamate moiety are potential candidates for application in ADEPT if enzymatic activation can be accelerated. The relatively low cytotoxicity of **DAU-GA1** compared to daunorubicin (table 2.3) and the facile hydrolysis of model compound *N*-phenyl *O*-β-D-glucuronyl carbamate (**8**, chart 2.4) makes the β-D-glucuronyl carbamate group an interesting specifier to be used in prodrug development. The research presented in the following chapters focuses on the synthetic approaches to and evaluation of β-D-glucuronyl carbamate-based prodrugs possessing an immolative spacer entity between drug and β-D-glucuronyl carbamate specifier to facilitate enzymatic prodrug activation.

2.7 Experimental part

2.7.1 Biological evaluation

Cytotoxicities

The cytotoxicities of the daunorubicin-phosphate and -sulfate analogs (table 2.2) were evaluated using a panel of 5 human tumor cell lines consisting of rhabdomyosarcoma cells (A-204), mammary carcinoma cells (MCF-7), bladder carcinoma cells (T-24), melanoma cells (IgR-37) and colon carcinoma cells (WiDr). For the β-D-glycosyl prodrugs (table 2.3), a panel consisting of MCF-7 and WiDr Cells, mammary carcinoma cells (EVSAT), ovarian carcinoma cells (IGROV), melanoma cells (M-19) and renal cancer cells (A-498) was used [46]. IC50 values were calculated from a dose-response curve which was obtained from cell-kill values at 12 different concentrations performed twice for each drug.

Enzymatic prodrug activation rates

The enzymatic activation rates of the β-D-glucuronyl-containing prodrugs were determined by incubation of 100 μM of prodrug in 0.1% BSA/PBS at pH = 6.8 [47] with 0.03 U/mL human β-glucuronidase at 37°C. The β-D-glucose and β-D-galactose based prodrugs were incubated with 0.3 U/mL bovine liver β-galactosidase [44] and the daunorubicin-sulfates were incubated with 1 μg/mL aryl sulfatase under the same conditions as the β-D-glucuronides. Samples were prepared and analyzed on reversed phase SiO_2-C_{18} HPLC as described [11b].

2.7.2 Chemistry

General

Daunorubicin hydrochloride **DAU**-HCl was a generous gift from Pharmachemie BV Haarlem. 2-(Methylsulfonyl)ethyl alcohol was kindly provided by Prof. G.I. Tesser. Chromatotron model 7924-T Harrison Research (Palo Alto, California, USA) equipped with plates (thickness 2 mm, diameter 8.5 cm) made form Merck silicagel 60 PF_{254} which contains gypsum (art. 7749) was used when circular chromatography is indicated. Reversed phase chromatography was performed with a liquid chromatography pump LC-410 (Kontron) using a pre-packed column (24 cm, diameter 11 mm) containing octadecylsilane (40-63 μm) (Merck, Darmstadt). Prior to use the RP-C_{18} column was equilibrated with demineralized water. ^{1}H NMR spectra at 400 MHz were obtained on a Bruker AM-400. Chemical shifts are expressed in ppm downfield from internal standard Me_4Si, the ^{31}P NMR spectra were recorded at 162 MHz using $(MeO)_3PO$ as external standard on a Bruker AM-400. Abbreviations are used as follows: 2-(methylsulfonyl)ethyl-: MSE-. All solvents were dried before use. MeCN, CH_2Cl_2 and Et_3N were dried by distillation over CaH_2, $CHCl_3$ and pyridine by distillation over $CaCl_2$ and THF by distillation over $LiAlH_4$ or over sodium. *i*-Pr_2NEt was dried over KOH pellets. In all cases demineralized H_2O was used.

Synthesis of daunorubicin-*N*-phosphate disodium salt (DAU-P1).

Daunorubicin-N-(bis[2-(methylsulfonyl)ethyl] phosphate) (**11**). To a stirred suspension of 340 mg (0.60 mmol) of **DAU**-HCl and 0.84 mL (10 equiv.) of Et_3N cooled to -40 °C was added dropwise a solution of 238 mg of bis[MSE]-phosphorochloridate **10** (1.2 equiv.) in MeCN. After 2 h, TLC (CH_2Cl_2/EtOH, 10/1) indicated completion of the reaction. After evaporation of the MeCN and the Et_3N under reduced pressure, the red residue was dissolved in $CHCl_3$ washed with 20 mL portions of saturated aqueous $NaHCO_3$ (3x), H_2O (2x) and brine and dried over Na_2SO_4. After evaporation of the $CHCl_3$, the unstable **11** which formed cyclic phosphate **12** upon standing, was immediately deprotected.

Daunorubicin-N-phosphate disodium salt (**DAU-P1**). Compound **11** obtained as above was dissolved in 40.0 mL of dioxane/MeOH 14/5 and 3.0 mL of 4 N NaOH (20 equiv.) in 12.0 mL of dioxane/MeOH 14/5. The resulting deep blue solution was quenched after 30 sec. until the blue colour had disappeared using solutions of 2 N and 0.1 N HCl successively. After this, the red solution was poured into 100 mL of an aqueous 0.1 M phosphate buffer pH = 7.5. The aqueous solution was diluted to *ca.* 400 mL with H_2O and washed with $CHCl_3$ (6x). The aqueous layer was concentrated under reduced pressure to *ca.* 250 mL and applied to a RP-C_{18} pre-packed column. The column was eluted with H_2O (250 mL) to remove the inorganic salts. Elution of the product from the column using 20 mL of MeCN/H_2O 1/1, evaporation of MeCN and lyophilizing the H_2O yielded 154 mg, 40% of pure **DAU-P1** as a red fluffy solid, mp 210-230°C. Anal.: calc. (found) for $C_{27}H_{28}NO_{13}PNa_2 \cdot 4{,}5\ H_2O$: C: 44.27 (44.28), H: 5.09 (5.09), N: 1.91 (1.96). MS (FAB$^+$) m/z = 688 ([M(with $P(O)O_2Na_2$)+H+2 H_2O]$^+$), 666 ([M(with $P(O)O_2NaH$)+H+2 H_2O]$^+$), 644 ([M(with

$P(O)O_2H_2$)+H+2 H_2O]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.12 (d, 3H, 5'-Me, *J* = 6.2 Hz), 1.54 (bd, 1H, 2'$_{eq}$-H, *J* = 9.8 Hz), 1.77 (bt, 1H, 2'$_{ax}$-H, *J* = 12.3 Hz), 2.15 (bd, 1H, 8$_{ax}$-H, *J* = 13.3), 2.20 (bd, 1H, 8$_{eq}$-H, *J* = 11.2 Hz), 2.24 (s, 3H, 9-C(O)Me), 2.91 (d, 1H, 10$_{eq}$-H, *J* = 16.2 Hz), 3.01 (d, 1H, 10$_{ax}$-H, *J* = 17.8 Hz), 3.10-3.55 (m, 2H, 3',4'-H), 3.97 (s, 3H, 4-OMe), 4.03 (q, 1H, 5'-H, *J* = 6.7 Hz), 4.96 (bs, 1H, 7-H), 5.18 (bs, 1H, 1'-H), 5.48 (bs, 1H, 3'-NH-), 7.60 (bs, 1H, 3-H), 7.79 (bs, 2H, 1,2-H). Due to the presence of H_2O in the $(CD_3)_2SO$, signals of -OH groups were not present in the spectrum. ^{31}P-NMR (162 MHz, proton-decoupled, $(CD_3)_2SO$) δ (ppm) = 8.11.

Synthesis of daunorubicin-*N*-sulfate sodium salt (DAU-S1).

0.23 mL Et_3N (4 equiv.) and 152 mg (2 equiv.) of Et_3N-SO_3 salt in 8.0 mL of dry $CHCl_3$ were added to 237 mg (0.420 mmol) of daunorubicin. After stirring the reaction mixture at ambient temperature overnight, another 2 equiv. of the Et_3N-SO_3 reagent were added and reaction was stirred at reflux temperature. After 1 h, *ca.* 90% of the starting material had disappeared (RP-C_{18} TLC, MeCN/H_2O 1/1) and the suspension was taken to dryness. To remove the unreacted daunorubicin, the red residue was dissolved in 100 mL of H_2O containing 2 mL of saturated aqueous $NaHCO_3$ and was washed with three portions of 50 mL $CHCl_3$. The aqueous solution of **DAU-S1** was purified on a RP-C_{18} column as described for **DAU-P1**. The thus obtained triethylammonium salt of the sulfate was converted to the sodium salt by eluting an aqueous solution of the product over a column containing cation exchange material (Na^+ form). The product fraction was lyophilized to furnish 188 mg, 71% of pure **DAU-S1** as a red fluffy solid, mp 188-191ºC. Anal.: calc. (found) for $C_{27}H_{28}NO_{13}SNa\cdot 3\ H_2O$: C: 47.44 (47.09), H: 5.01 (4.97), N: 2.05 (2.36), S: 4.69 (4.40). MS (FAB$^+$) *m*/*z* = 652 ([M+Na]$^+$), 630 ([M+H]$^+$). ^{1}H-NMR (400 MHz, $CDCl_3$) of Et_3N^+H salt δ (ppm) = 1.13 (d, 3H, 5'-Me, *J* = 6.1 Hz), 1.25-1.35 (m, 15H, *Et*$_3$N$^+$H), 1.82 (dt, 1H, 2'$_{ax}$-H, *J* = 12.5 Hz *J* = 4.5 Hz), 1.86 (dd, 1H, 2'$_{eq}$-H, *J* = 13.4 Hz *J* = 3.8 Hz), 2.07 (dd, 1H, 8$_{ax}$-H, *J* = 14.8 Hz *J* = 4.1 Hz), 2.35 (d, 1H, 8$_{eq}$-H, *J* = 14.8 Hz), 2.41 (s, 3H, 9-C(O)Me), 2.97 (d, 1H, 10$_{ax}$-H, *J* = 18.8 Hz), 3.00-3.15 (m, 2H, 3'-NH- 4'-OH), 3.20 (dd, 1H, 10$_{eq}$-H, *J* = 18.9 Hz *J* = 1.6 Hz), 3.63 (bd, 1H, 4'-H, *J* = 6.4 Hz), 3.85 (bs, 1H, 3'-H), 4.07 (s, 3H, 4-OMe), 4.15 (q, 1H, 5'-H, *J* = 6.8 Hz), 4.71 (s, 1H, 9-OH), 5.28 (d, 1H, 7-H, *J* = 1.8 Hz), 5.52 (d, 1H, 1'-H, *J* = 3.5 Hz), 7.39 (d, 1H, 3-H, *J* = 8.4 Hz), 7.78 (t, 1H, 2-H, *J* = 8.1 Hz), 8.03 (d, 1H, 1-H, *J* = 7.5 Hz), 13.29 (s, 1H, 11-OH), 13.95 (s, 1H, 6-OH).

Synthesis of *N*-3'-daunorubicinyl *O*-(2-(methylsulfonyl)ethyl) carbamate (15).

To a stirred suspension of 100 mg (0.177 mmol) of **DAU**-HCl and (61 μL, 2.0 equiv.) of *i*-Pr_2NEt in 10 mL of DMF, 52 mg (1.1 equiv.) of MSE 1-hydroxysuccinimidyl carbonate **14** was added. After 0.5 h of stirring, reaction was complete and the mixture was diluted with 150 mL of EtOAc and washed successively with aqueous 0.5 N $KHSO_4$ (2x), H_2O, saturated aqueous $NaHCO_3$ (2x), H_2O and brine. The organic fraction was dried over Na_2SO_4 and taken to dryness. The crude product was purified in two runs by circular chromatography using respectively CH_2Cl_2/EtOH 10/1 and 20/1 as the eluent. The product fraction was evaporated, the red residue was sonicated in *i*-Pr_2O and filtered of to yield 104 mg, 87% of **15** as an orange powder, mp 155ºC. Anal.: calc. (found) for $C_{31}H_{35}NO_{14}S\cdot 1\ H_2O$: C: 53.75 (53.52), H: 5.36 (5.17), N: 2.01 (2.04), S: 4.61 (4.60). MS (FAB$^+$) *m*/*z* = 700 ([M+Na]$^+$), 778 ([M+H]$^+$). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.30 (d, 3H, 5'-Me, *J* = 6.8 Hz), 1.78 (dd, 1H, 2'$_{ax}$-H, *J* = 12.9 Hz *J* = 4.0 Hz), 1.90 (dd, 1H, 2'$_{eq}$-H, *J* = 13.1 *J* = 4.8), 2.12 (dd, 1H, 8$_{ax}$-H, *J* = 14.8, *J* = 4.1 Hz), 2.31 (d, 1H, 8$_{eq}$-H, *J* = 14.8 Hz), 2.42 (s, 3H, 9-C(O)Me), 2.92 (d, 1H, 10$_{ax}$-H, *J* = 18.6), 2.94 (s, 3H, -SO_2Me), 3.20-3.35 (m, 3H, 10$_{eq}$-H, -C*H*$_2$$SO_2$Me), 3.67 (bs, 1H, 4'-H), 3.88 (bs, 1H, 3'-H), 4.07 (s, 3H, 4-OMe), 4.21 (q, 1H, 5'-H *J* = 6.6 Hz), 4.44 (s, 1H, 9-OH), 4.45-4.50 (m, 2H, -C*H*$_2$$CH_2SO_2$Me), 5.23 (d, 1H, 3'-NH-, *J* = 8.8 Hz), 5.26 (d, 1H, 7-H, *J* = 1.8 Hz), 5.50 (d, 1H, 1'-H, *J* = 3.5 Hz), 7.39 (d, 1H, 3-H, *J* = 8.4 Hz), 7.78 (t, 1H, 2-H, *J* = 8.0 Hz), 8.03 (d, 1H, 1-H, *J* = 7.6 Hz), 13.27 (s, 1H, 11-OH), 13.99 (s, 1H, 6-OH).

Synthesis of daunorubicin-4′-*O*-phosphate (DAU-P2).

Daunorubicin-N-(carbonyl-oxy-2-(methylsulfonyl)ethyl) -4′-O-(bis[2-(methylsulfonyl)ethyl] phosphate) (**17**). 150 mg (0.222 mmol) of the 3'-amino protected daunorubicin **15**, 167 mg (2.0 equiv.) of phosphitilating reagent **16** and 17 mg (1.1 equiv.) of 1-*H*-tetrazole were dissolved in CH_2Cl_2 under an argon atmosphere and heated to reflux. Conversion of the starting material to daunorubicin-4'-phosphite was monitored by TLC (SiO_2, CH_2Cl_2/EtOH 10/1). After 30 min, additional portions of 0.1 equiv. of both phosphite and tetrazole were added. After 1 h, almost all of the starting material had disappeared. The reaction mixture was cooled to -67 ºC and *m*-CPBA (115 mg, 3.0 equiv.), dissolved in 10 mL CH_2Cl_2 was added. The

phosphite was easily oxidized to the protected phosphate **17**. The reaction mixture was diluted with 150 mL of CH_2Cl_2 and washed with 10% aqueous Na_2SO_3 (4x 50 mL), saturated aqueous $NaHCO_3$ (2x), H_2O (2x) and brine successively. The organic layer was dried with Na_2SO_4 and the solvent was evaporated.

Daunorubicin-4'-O-phosphate (**DAU-P2**). The **17** obtained as above was dissolved in 20 mL of dioxane /MeOH 14/5 and 1.66 mL of 4 N aqueous NaOH (10 equiv. per protective group) in 11.7 mL of dioxane/MeOH 14/5 was added in one portion while vigorously stirring the mixture. After 1 min, the resulting deep blue solution was quenched with an aqueous 1.0 N AcOH solution until the blue colour turned red and poured into 100 mL of a 0.1 N aqueous phosphate buffer pH = 7.0. The resulting aqueous solution of **DAU-P2** was purified on a reversed phase column as described for **DAU-P1** to obtain 104 mg, 74% of **DAU-P2** as a red fluffy solid, mp >350ºC. Anal.: calc. (found) for $C_{27}H_{29}NO_{13}PNa{\cdot}4\ H_2O$: C: 46.23 (46.51), H: 5.32 (5.09), N: 2.00 (2.06). MS (FAB$^+$) *m/z* = 644 ([M(with $P(O)O_2H_2$)+H+2 H_2O]$^+$), 630 ([M(with $P(O)O_2NaH$)+H]$^+$), 608 ([M(with $P(O)O_2H_2$)+H]$^+$). In the NMR-spectrum, signals were strongly broadened [49]. ^{1}H-NMR (400 MHz, (D_2O) δ (ppm) = 1.21 (d, 3H, 5'-Me, *J* = 6.5 Hz), 1.80-1.90 (m, 2H, 2'$_{ax}$-H 2'$_{eq}$-H), 2.00-2.15 (m, 2H, 8$_{ax}$-H 8$_{eq}$-H), 2.33 (s, 3H, 9-C(O)Me), 2.44 (bd, 1H, 10$_{ax}$-H, *J* = 17.2), 2.68 (bd, 1H, 10$_{ax}$-H, *J* = 17.2 Hz), 2.91 (d, 1H, 10$_{eq}$-H, *J* = 16.2 Hz), 3.60-3.65 (m, 1H, 3'-H), 3.69 (s, 3H, 4-OMe), 4.16 (bd, 1H, 5'-H, *J* = 7.5 Hz), 4.40-4.65 (m, 3H, 7-H 4'-H 3'-NH_3^+), 5.30 (bs, 1H, 1'-H), 7.11 (bs, 2H, 1,3-H), 7.39 (bs, 1H, 2-H). Due to the presence of H_2O, signals of -OH groups were not present in the spectrum. ^{31}P-NMR (162 MHz, proton-decoupled, (D_2O) δ (ppm) = 4.35.

Synthesis of daunorubicin-4'-*O*-sulfate (DAU-S2).

Daunorubicin-N-carbonyl-oxy-2-(methylsulfonyl)ethyl -4'-O-sulfate (**18**). 0.2 mL of $ClSO_3H$ was added to 10 mL of dry pyridine under a stream argon. 2.0 mL of this 0.3 mmol/mL pyr-SO_3 solution was transferred to a solution of 184 mg (0.272 mmol) of **15** in 10 mL dry pyridine. After 2 h, an additional portion of 2.0 mL of the sulfate reagent was added. Progress of the reaction was monitored on TLC (RP-C_{18} MeCN/H_2O 1/1). When almost all of the starting material had disappeared (*ca.* 2 h), 1.0 mL of a saturated aqueous $NaHCO_3$ solution in 100 mL of H_2O were added. The solution was washed with $CHCl_3$ and purified as described for **DAU-P1**.

Daunorubicin-4'-O-sulfate (**DAU-S2**). The protected sulfate **18** was dissolved in 7.0 mL of dioxane/MeOH 14/5. 0.6 mL of 4 N aqueous NaOH (10 equiv.) in 4.4 mL of dioxane/MeOH 14/5 was added in one portion. After vigorously stirring for 1 min, the mixture was quenched until the blue colour turned red with a 2.0 N aqueous AcOH solution, and poured into 100 mL of a 0.1 N aqueous phosphate buffer pH = 7.5. The aqueous solution was washed with $CHCl_3$ and purified using a reversed phase column as described for **DAU-P1**. Lyophilisation of the product fraction from the RP-C_{18} column afforded 85 mg, 51% of **DAU-S2** as a red fluffy solid, mp >350ºC. Anal.: calc. (found [48]) for $C_{27}H_{29}NO_{13}S{\cdot}4\ H_2O$: C: 47.72 (47.26), H: 5.49 (4.70), N: 2.06 (2.10), S: 4.72 (5.13). MS (FAB$^+$) *m/z* = 630 ([M+Na]$^+$), 608 ([M+H]$^+$). Due to the poor solubility of the sulfate during work-up, a considerable amount of the product was lost. In the NMR-spectrum, signals were broadened [49]. ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.17 (d, 3H, 5'-Me, *J* = 6.0 Hz), 1.69 (bs, 1H, 2'$_{ax}$-H), 1.82 (bs, 1H, 2'$_{eq}$-H), 2.05-2.15 (m, 2H, 8$_{ax}$-H 8$_{eq}$-H), 2.26 (s, 3H, 9-C(O)Me), 2.89 (d, 1H, 10$_{ax}$-H, *J* = 19.6 Hz), 2.94 (d, 1H, 10$_{eq}$-H, *J* = 19.6 Hz), 3.97 (s, 3H, 4-OMe), 4.20 (bs, 1H,4'-H), 4.24 (d, 1H, 5'-H, *J* = 6.0 Hz), 4.95 (bs, 1H, 7-H), 5.28 (bs, 1H, 1'-H), 5.41 (bs, ±1H, 3'-NH_3^+), 7.62 (bs, 1H, 3-H), 7.89 (bs, 2H, 1,2-H). Due to the presence of H_2O in the $(CD_3)_2SO$, signals of -OH groups were not present in the spectrum. The 3'-H signal was overlapped by H_2O signal [50].

Synthesis of *N*-3'-daunorubicinyl *O*-β-D-glucuronyl carbamate (DAU-GA1).

Diastereoselective formation of 1-O-β-trimethylsilyl methyl 2,3,4-tri-O-acetyl D-glucuronic acid (**40**). To a solution of 100 mg (299 mmol) of the anomerically unprotected D-glucuronic acid **27** in 2.0 mL of THF was added 114 μL (2.0 equiv.) of *N,N*-diethyl trimethylsilyl amine (**39b**) at 0 ºC under an argon atmosphere. After stirring the reaction mixture for 48 h at 0 ºC, the starting material had disappeared (TLC, SiO_2, Et_2O). After removal of the THF, diethyl amine and excess silylating reagent under reduced pressure, **40** was obtained in 90% yield (determined by ^{1}H-NMR). ^{1}H-NMR spectrum according to literature [40]. the unstable 1-*O*-β-trimethylsilyl D-glucuronide was immediately used in the next reaction. Using different silylating reagents and reaction conditions (see table 2.1), the anomeric composition of the

silylated product was evaluated using the integrated signals of 5-H from both anomers in the 100 MHz ^{1}H-NMR spectrum in $CDCl_3$ (δ 5-H_α = 4.45 ppm, δ 5-H_β = 3.99 ppm).

N-3'-Daunorubicinyl O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**38***β*). To 1-*O*-*β*-trimethylsilyl D-glucuronide **40** obtained as described above in 5 mL of MeCN, 64 mg (1,2 equiv.) of chloroformate **33** and 10 mg (0.25 equiv.) of anhydrous $ZnCl_2$ were added. When most of **40** had disappeared, 48 μL (2.0 equiv.) of pyridine were added. After 0.5 h, 254 mg (1.5 equiv.) of **DAU**-HCl and 156 μL (3.0 equiv.) of *i*-Pr_2NEt were added. The reaction mixture was diluted with 200 mL of CH_2Cl_2 and washed with a 5% aqueous solution of $KHSO_4$ (3x), H_2O and saturated aqueous $NaHCO_3$ (2x), and with brine successively. The organic layer was dried over Na_2SO_4 and evaporated. The crude **38***β* was purified by circular chromatography in three successive sessions using CH_2Cl_2/EtOH 10/1, 20/1 and 30/1 respectively. Evaporation of the product fractions, sonification of the residue in *i*-Pr_2O and filtration yielded 115 mg (48%) of **38***β*, mp 160-162ºC. Anal.: calc. (found) for $C_{41}H_{45}NO_{21}\cdot 1\ H_2O$: C: 54.37 (54.67), H: 5.23 (5.34), N: 1.55 (1.50). ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.29 (d, 1H, 5'-Me, *J* = 6.6 Hz), 1.76 (dt, 1H, 2'$_{ax}$-H, *J* = 13.2 Hz *J* = 4.0 Hz), 1.89 (dd, 1H, 2'$_{eq}$-H, *J* = 13.4 Hz *J* = 4.8 Hz), 1.99 (s, 6H, 2 -OAc), 2.01 (s, 3H, -OAc), 2.10 (dd, 1H, 8$_{ax}$-H, *J* = 14.8 Hz *J* = 4.1 Hz), 2.28 (d, 1H, 8$_{eq}$-H, *J* = 14.8 Hz), 2.39 (s, 3H, 9-C(O)Me), 2.46 (s, 1H, 4'-OH), 2.93 (d, 1H, 10$_{ax}$-H, *J* = 18.9 Hz), 3.23 (d, 1H, 10$_{eq}$-H, *J* = 19.1 Hz), 3.64 (bd, 1H, 4'-H, *J* = 6.4 Hz), 3.68 (s, 3H, -CO_2Me), 3.87 (bs, 1H, 3'-H), 4.07 (s, 3H, 4-OMe), 4.08 (d, 1H, Gluc5-H, *J* = 9.9 Hz), 4.19 (q, 1H, 5'-H, *J* = 6.4 Hz), 4.42 (s, 1H, 9-OH), 5.08 (t, 1H, Gluc2-H, *J* = 8.7 Hz), 5.16 (t, 1H, Gluc4-H, *J* = 9.6 Hz), 5.20-5.30 (m, 1H, 7-H), 5.27 (t, 1H, Gluc3-H, *J* = 9.3 Hz), 5.37 (d, 1H, 3'-NH-, *J* = 8.6 Hz), 5.48 (d, 1H, 1'-H, *J* = 3.5 Hz), 5.62 (d, 1H, Gluc1-H, *J* = 8.0 Hz), 7.38 (d, 1H, 3-H, *J* = 8.4 Hz), 7.77 (t, 1H, 2-H, *J* = 8.0 Hz), 8.03 (d, 1H, 1-H, *J* = 7.7 Hz), 13.26 (s, 1H, 11-OH), 13.97 (s, 1H, 6-OH).

N-3'-Daunorubicinyl O-β-D-glucuronyl carbamate (**DAU-GA1**). 42 mg of **38***β* (47.4 μmol) was dissolved in 1.90 mL of a 0.25 N solution of LiOH in MeOH/H_2O 3/1 (10 equiv.). The resulting deep blue solution was stirred at 0 ºC under an argon atmosphere while the course of the reaction was monitored on TLC (RP-C_{18} MeCN/H_2O 1/1). After 20 min, the reaction mixture was diluted with 50 mL of H_2O and the LiOH solution was neutralized with *ca* 1 g of amberlite cation exchange material (H^+ form) whereupon the blue mixture turned to red. After the amberlite had been removed by filtration, 0.5 mL of saturated aqueous $NaHCO_3$ was added. The prodrug was purified on a reversed phase column as described for **DAU-P1** to afforded 34 mg, 93% of **DAU-GA1**, mp 178ºC. Anal.: calc. (found) for $C_{34}H_{36}NO_{18}Na\cdot 4\ H_2O$: C: 48.52 (48.08), H: 5.27 (4.87), N: 1.66 (1.75). MS (FAB$^+$) *m/z* = 792 ([M+Na]$^+$), 770 ([M+H]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.12 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.54 (dd, 1H, 2'$_{eq}$-H, *J* = 11.6 Hz *J* = 3.4 Hz), 1.84 (dt, 1H, 2'$_{ax}$-H, *J* = 11.5 Hz *J* = 3.4 Hz), 2.08 (dd, 1H, 8$_{ax}$-H, *J* = 13.7 Hz *J* = 5.3 Hz), 2.20 (bd, 1H, 8$_{eq}$-H, *J* = 11.2 Hz), 2.25 (s, 3H, 9-C(O)Me), 2.90 (d, 1H, 10$_{eq}$-H, *J* = 18.2 Hz), 2.96 (d, 1H, 10$_{ax}$-H, *J* = 18.2 Hz), 3.00-3.45 (m, 5H, 4'-H Gluc2,3,4,5-H), 3.72 (m, 1H, 3'-H), 3.96 (s, 3H, 4-OMe), 4.16 (q, 1H, 5'-H, *J* = 6.6 Hz), 4.76 (d, 1H, -OH), 4.90 (t, 1H, 7-H, *J* = 4.4 Hz), 5.00 (s, 1H, -OH), 5.05 (s, 1H, -OH), 5.09 (d, 1H, Gluc1-H, *J* = 8.1 Hz), 5.21 (s, 1H, 1'-H), 5.55 (s, 1H, 9-OH), 5.77 (s, 1H, -OH), 6.11 (s, 1H, -OH), 6.93 (d, 1H, 3'-NH-, *J* = 8.0 Hz), 7.60 (dd, 1H, 3-H, *J* = 6.3 Hz *J* = 3.3 Hz), 7.80-7.90 (m, 2H, 1,2-H), 13.23 (s, 1H, 11-OH), 14.01 (s, 1H, 6-OH).

Synthesis of *N*-3'-daunorubicin *O*-*β*-D-glucosyl carbamate (DAU-GsA1).

N-3'-Daunorubicinyl O-β-(2,3,4,6-tetra-O-acetyl D-glucosyl) carbamate (**43**). To a solution of 126 mg (4 equiv.) of chloroformate **33** and 115 μL (8 equiv.) of pyridine in 15 mL of THF was added 62 mg (0.178 mmol) of 2,3,4,6-tetra-*O*-acetyl *β*-D-glucose **41**. The mixture was stirred for 2 h and 120 mg (1.2 equiv.) of **DAU**-HCl and 74 μL (2.4 equiv.) of *i*-Pr_2NEt in 5 mL of DMF were added. After 2 h, the reaction mixture was worked up as described for **38***β* to obtain 96 mg, 60% of **43** as amorphous red crystals, mp 152-155ºC. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.28 (d, 3H, 5'-Me, *J* = 6.6 Hz), 1.70-1.95 (m, 3H, 2'$_{eq}$-H 2'$_{ax}$-H 4'-OH), 1.99, 2.00, 2.02 and 2.03 (4s, 12H, 4 -OAc), 2.10 (dd, 1H, 8$_{ax}$-H, *J* = 14.8 Hz *J* = 3.8 Hz), 2.30 (d, 1H, 8$_{eq}$-H, *J* = 14.8 Hz), 2.38 (s, 3H, 9-C(O)Me), 2.90 (d, 1H, 10$_{ax}$-H, *J* = 18.8 Hz), 3.22 (d, 1H, 10$_{eq}$-H, *J* = 18.8 Hz), 3.63 (bs, 1H, 4'-H), 3.75-3.85 (m, 2H, 3'-H Gluc5-H), 4.06 (s, 3H, 4-OMe), 4.05 (d, 1H, Gluc6-H_aH$_b$-, *J* = 11.6 Hz), 4.21 (q, 1H, 5'-H, *J* = 6.5 Hz), 4.24 (dd, 1H, Gluc6-H$_a$$H_b$-, *J* = 11.4 Hz *J* = 4.3 Hz), 4.46 (s, 1H, 9-OH), 5.05-5.35 (m, 5H, 7-H 3'-NH- Gluc2,3,4-H), 5.46 (d, 1H, 1'-H, *J* = 3.3 Hz), 5.60 (d, 1H, Gluc1-H, *J* = 8.0 Hz), 7.37 (d, 1H, 3-H, *J* = 8.6 Hz), 7.78 (t, 1H, 2-H, *J* = 8.0 Hz), 8.01 (d, 1H, 1-H, *J* = 7.5 Hz), 13.28 (s, 1H, 11-OH), 13.98 (s, 1H, 6-OH).

N-3'-Daunorubicinyl O-β-D-glucosyl carbamate (**DAU-GsA1**). 32 mg (0.035 mmol) of **43** was dissolved in 2.84 mL (10 equiv.) of a 0.125 N solution of LiOH in MeOH/H_2O 3/1 and stirred under an argon atmosphere at 0ºC. After 10 min, deprotection was complete as judged by TLC (RP-C_{18} MeCN/H_2O 1/1) and 10 mL of H_2O and 1 g of amberlite cation exchange material (H^+ form) were added. After the deep blue solution had turned red, the amberlite was filtered off, the residue was washed with THF and the filtrate was concentrated under reduced pressure. After that, 2 mL of MeCN were added and the solution was transferred to a RP-C_{18} column. The column was washed with 200 mL of MeCN/H_2O 1/5 and the product was eluted with MeCN/H_2O 1/1, MeCN was evaporated and the aqueous solution was lyophilized to yield 25 mg, 97% of **DAU-GsA1** as a red fluffy solid, mp 163-170ºC. Anal.: calc. (found) for $C_{34}H_{39}NO_{17}\cdot 3\ H_2O$: C: 51.84 (51.36), H: 5.76 (5.52), N: 1.78 (2.42). MS (FAB$^+$) *m/z* = 756 ([M+Na]$^+$), 734 ([M+H]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.13 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.45 (dd, 1H, 2'$_{eq}$-H, *J* = 11.8 Hz *J* = 4.0 Hz), 1.87 (dt, 1H, 2'$_{ax}$-H, *J* = 11.6 Hz *J* = 4.8 Hz), 2.05 (dd, 1H, 8$_{ax}$-H, *J* = 14.2 Hz *J* = 5.7 Hz), 2.20 (d, 1H, 8$_{eq}$-H, *J* = 14.2 Hz), 2.26 (s, 3H, 9-C(O)Me), 2.90 (s, 2H, Gluc6-H_2), 3.00-3.80 (m, 8H, 10$_{eq}$-H 10$_{ax}$-H 3',4'-H, Gluc2,3,4,5-H), 3.96 (s, 3H, 4-OMe), 4.17 (q, 1H, 5'-H, *J* = 6.6 Hz), 4.46 (s, 1H, -OH), 4.69 (s, 1H, -OH), 4.90 (s, 1H, 7-H), 5.08 (s, 1H, -OH), 5.15 (d, 1H, Gluc1-H, *J* = 8.1 Hz), 5.21 (s, 1H, 1'-H), 5.49 (s, 1H, 9-OH), 6.92 (d, 1H, 3'-NH-, *J* = 7.8 Hz), 7.62 (t, 1H, 3-H, *J* = 4.6 Hz), 7.80-7.85 (m, 2H, 1,2-H), 13.25 (s, 1H, 11-OH), 14.07 (s, 1H, 6-OH).

Synthesis of *N*-3'-daunorubicinyl *O*-β-D-galactosyl carbamate (DAU-GlA1).

N-3'-*Daunorubicinyl O-β-(2,3,4,6-tetra-O-acetyl D-galactosyl) carbamate* (**45**).

This compound was prepared in a three step reaction sequence from **44** according to the method described in section 5.2 (scheme 5.6, section 5.2, compound **37b**).

N-3'-*Daunorubicinyl O-β-D-galactosyl carbamate* (**DAU-GlA1**). 56 mg (0.062 mmol) of **45** was dissolved in 3.0 mL (6 equiv.) of a 0.125 N solution of LiOH in MeOH/H_2O 3/1 and stirred under an argon atmosphere at 0ºC. After 10 min, deprotection was complete as judged by TLC (RP-C_{18} MeCN/H_2O 1/1) and the reaction mixture was processed as described for **DAU-GsA1** to yield 36 mg, 79% of **DAU-GlA1** as a red fluffy solid, mp 163-170ºC. Anal.: calc. (found) for $C_{34}H_{39}NO_{17}\cdot 3\ H_2O$: C: 51.84 (52.13), H: 5.76 (5.30), N: 1.78 (2.09). MS (FAB$^+$) *m/z* = 756 ([M+Na]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.12 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.46 (dd, 1H, 2'$_{eq}$-H, *J* = 12.3 Hz *J* = 4.0 Hz), 1.84 (dd, 1H, 2'$_{ax}$-H, *J* = 13.2 Hz *J* = 3.8 Hz), 2.08 (dd, 1H, 8$_{ax}$-H, *J* = 14.2 Hz *J* = 5.3 Hz), 2.18 (d, 1H, 8$_{eq}$-H, *J* = 11.1 Hz), 2.25 (s, 3H, 9-C(O)Me), 2.93 (s, 2H, Gal6-H_2), 2.85-3.80 (m, 8H, 10$_{eq}$-H 10$_{ax}$-H, 3',4'-H, Gal2,3,4,5-H), 3.96 (s, 3H, 4-OMe), 4.17 (q, 1H, 5'-H, *J* = 6.6 Hz), 4.42 (d, 1H, -OH, *J* = 3.1 Hz), 4.53 (bs, 1H, -OH), 5.71 (d, 1H, -OH, *J* = 5.4 Hz), 4.75-4.95 (m, 3H, 7-H 2 -OH), 5.11 (d, 1H, Gal1-H, *J* = 7.9 Hz), 5.21 (d, 1H, 1'-H, *J* = 2.0 Hz), 5.51 (s, 1H, 9-OH), 6.89 (d, 1H, 3'-NH-, *J* = 8.0 Hz), 7.61 (t, 1H, 3-H, *J* = 7.6 Hz), 7.85-7.90 (m, 2H, 1,2-H), 13.24 (s, 1H, 11-OH), 14.00 (s, 1H, 6-OH).

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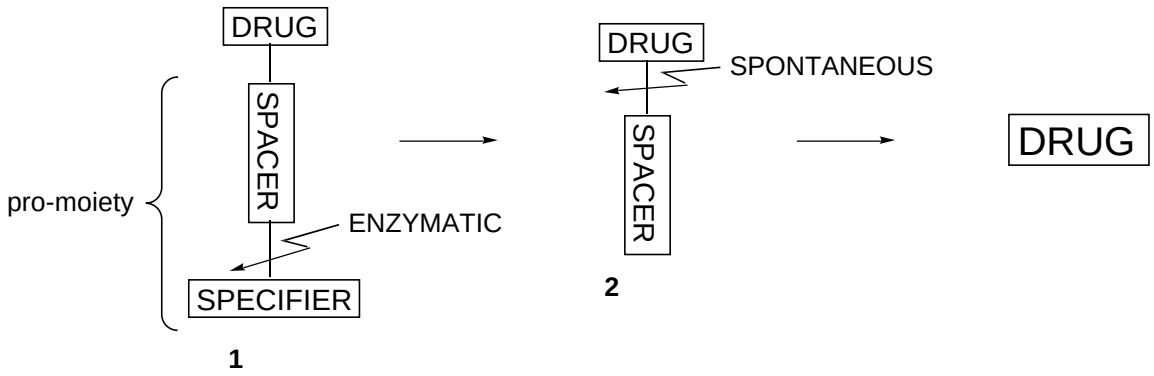
3 Daunorubicin-Spacer-β-D-Glucuronyl Carbamates as Prodrugs [1]

Spacer Elimination by Ring-closure

3.1 Introduction

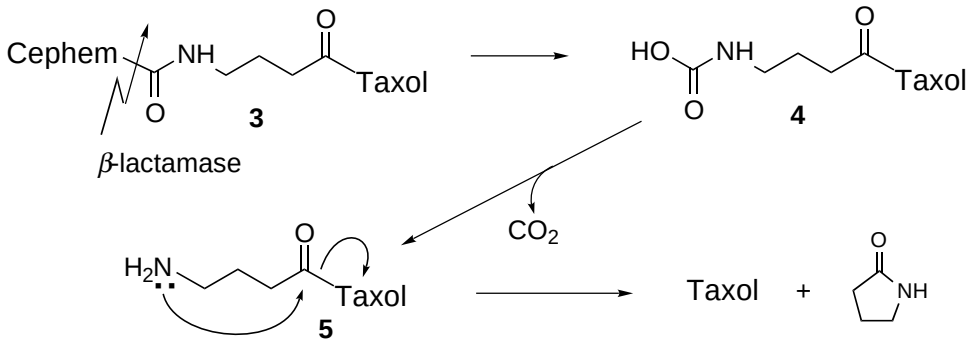
Important prerequisites of a prodrug to be applicable in ADEPT are *i.* low toxicity compared to the parent drug, *ii.* stability in plasma, *iii.* ready activation by the matching mAb-enzyme conjugate (for a more comprehensive overview of important prodrug characteristics, see chapter 1). As was concluded in chapter 2, among the prodrugs containing a phosphate- sulfate- β-D-glucosyl carbamate- or β-galactosyl- carbamate specifier, those having a β-D-glucuronyl carbamate specifier are promising for the use in ADEPT. In this respect, *N*-3'-daunorubicinyl *O*-β-D-glucuronyl carbamate **DAU-GA1** (chart 2.5) displayed a strongly reduced cytotoxicity compared to daunorubicin and was stable in serum. Although **DAU-GA1** was activated to the parent drug by β-glucuronidase, the rate was too low to fulfil the requirements for effective use in ADEPT. One possible way to improve the rate of hydrolysis of the β-D-glucuronyl specifier is the incorporation of a spacer moiety between daunorubicin and the β-D-glucuronyl carbamate specifier group. A general representation of prodrugs in which the pro-moiety consists of a spacer and a specifier is given in scheme 3.1. According to this scheme, after enzymatic hydrolysis of the specifier group of the intact prodrug **1**, elimination of the spacer from the spacer-drug molecule **2** is promoted and the free drug is generated. The use of such a three-component prodrug system enables optimization of enzymatic activation characteristics merely by structural variation of the spacer.



Scheme 3.1 *Spacer containing prodrugs.*

In the literature, two major types of self-eliminating (immolative) spacers are investigated. In this chapter, prodrugs containing a spacer which is eliminated by ring

closure are described. Chapter 4 deals with prodrugs having a spacer eliminated by a 1,6-elimination process.
An example of a prodrug containing a self-eliminating spacer is depicted in scheme 3.2. The specifier of compound **3** [2] is a cephalosporin derivative which is hydrolyzed by *β*-lactamase and upon this enzymatic triggering step, the resulting taxol-γ-aminobutyric acid **5** reverts to free taxol in a half-life time of approximately 6 hours at pH = 7.4 and 25°C. Earlier, taxol derivative **5** was prepared [3] which was found to be an unstable compound because of the intramolecular displacement reaction depicted in scheme 3.2.

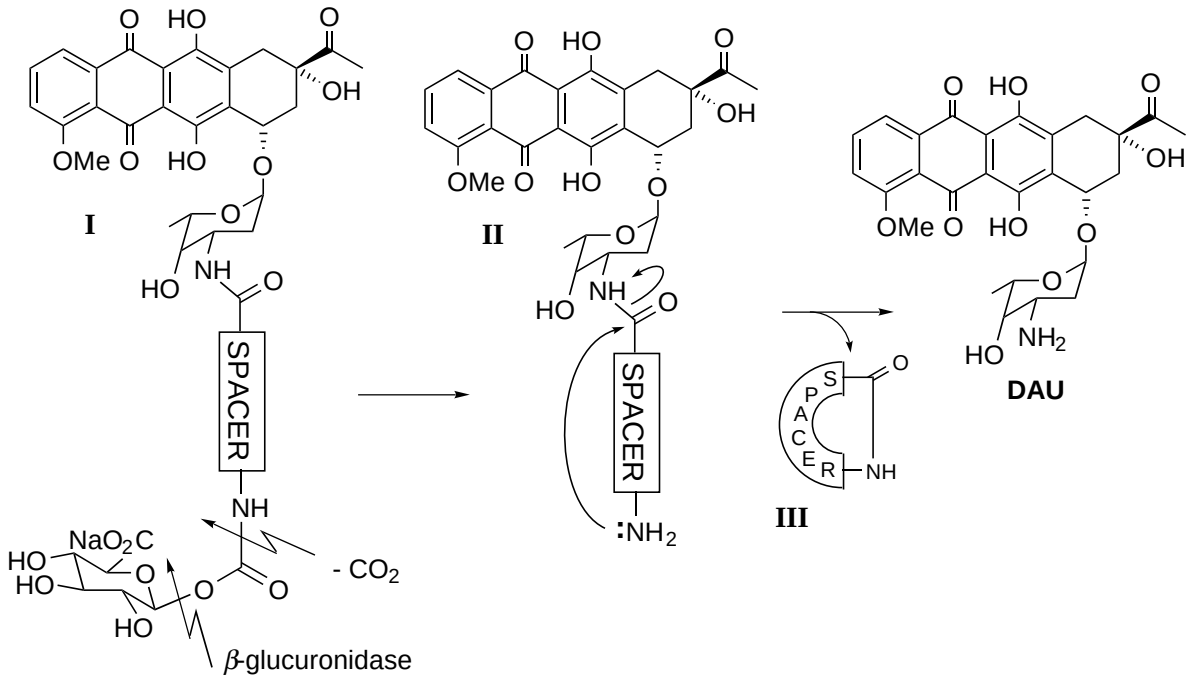


Scheme 3.2 *Taxol prodrug **3** with immolative cyclization spacer.*

3.2 Target compounds

3.2.1 General structure

The designed cycloeliminative spacer containing target molecules **I** are all based on the *β*-D-glucuronyl carbamate element (scheme 3.3). Compared to a *β*-D-glucuronyl ether group, the *β*-D-glucuronyl carbamate entity is expected to be more prone to hydrolysis

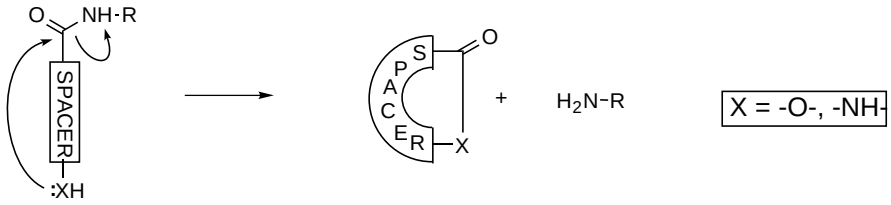


Scheme 3.3 *General representation of target compounds and their activation pathway to the free drug.*

than a conventional *O*-*β*-D-glucuronyl group (Indication for this supposition is given in chapter 2 by comparing rates of enzymatic hydrolysis of compounds in **7** {R = -*p*-nitrophenyl} and **8** chart 2.4). Moreover, after cleavage of the *β*-D-glucuronyl group of compounds **I** and loss of a molecule of CO_2, a primary amino group on spacer-drug molecule **II** is generated. This amine function may trigger the cycloelimination of the spacer. Prodrugs are designed in such a manner that elimination of the spacer of **II** should be a spontaneous process. The generation of the free drug from prodrugs **I** are dependent on: *i.* rate of enzymatic hydrolysis of the *β*-D-glucuronyl group, and *ii.* rate of the intramolecular cyclization reaction of the spacer entity leading to expulsion of daunorubicin. Loss of CO_2 from **II** will be instantaneous. Both the rates of the enzyme-mediated *β*-D-glucuronyl scission and that of the immolative spacer elimination are a function of the nature of the spacer. The enzyme-dependent factor is relatively unpredictable since the steric environment of the active site of human *β*-glucuronidase is not known.

3.2.2 Selection of the cyclisable spacer

The propensity of γ- and δ-amino and -hydroxy amides to undergo intramolecular cyclization is well documented in the literature [4] .(see scheme in table 3.1). This concept has found application in the fields of synthetic peptide chemistry [5] and protective group chemistry [6] as well as in prodrug design [7]. The kinetics and mechanism of cyclization of the above mentioned compounds have been studied in detail [8]. An excerpt of rates of ring-closure reactions of various hydroxy- and amino-amides is given in table 3.1.



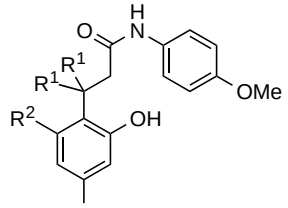
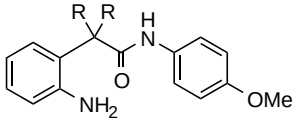
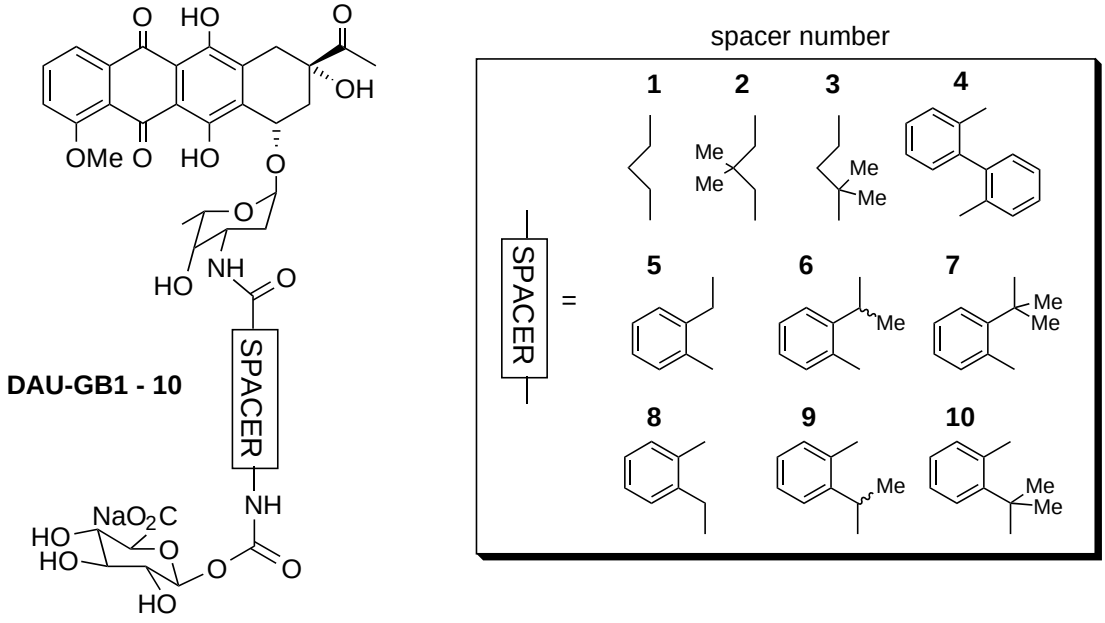
Reference	Compound	Cyclization $t_{1/2}$ (min)		Conditions
[7a]		(R^1, R^2 = -H)	$27 \cdot 10^3$	pH = 7.5, 30°C
		(R^1 = -Me, R^2 = -H)	630	pH = 7.5, 30°C
		(R^1, R^2 = -Me)	65.4	pH = 7.5, 30°C
[7d]		(R = -H, -H)	260	pH = 2.4, 37°C
		(R = -H, -Me)	16	pH = 2.4, 37°C
		(R = -Me, -Me)	0.07	pH = 2.4, 37°C
		(R = -Me, -Me)	72	pH = 6.8, 37°C

Table 3.1 *Cyclization half-lives of some hydroxy- and amino-amides.*

Cohen and co-workers published an extensive series of papers [9] describing the influence of substituents on rate constants of intramolecular cyclization reactions of hydroxy- and amino- esters, amides and acids. The designation "Stereopopulation Control" was introduced by these investigators to refer to the influence of the preorganization of a molecule on the cyclization rate.
As can be concluded from the data in table 3.1, rates of cyclization are markedly enhanced by methyl substitution at proper positions. In this context, prodrugs **GB1-10** (chart 3.1) are chosen to study glucuronide hydrolysis and the cyclization of the spacer leading to lactam **III** and the free drug daunorubicin (scheme 3.3). All glycosylated prodrugs in this thesis are named using the nomenclature system described in chart 3.1. It should be noted that the rate of cyclization of amino amides is relatively insensitive to the electronic environment of the leaving amine [5a, 7d, 8a] in the amido group. Therefore, it was expected that the poor leaving ability of the 3'-*N*-daunorubicinyl group would not impede the intramolecular eliminative cyclization reaction. The mechanism of the cyclization reaction is treated in more detail in section 3.4.



nomenclature of glycosylated prodrugs:

anthracycline: **DAU** = daunorubicin
DOX = doxorubicin (Ch. 4)
IDA = idarubicin (Ch. 4)

specifier: **G** = β-D-glucuronyl carbamate
Gs = β-D-glucosyl carbamate (Ch. 4)
Gl = β-D-galactosyl carbamate (Ch. 4)

specifier drug spacer type spacer number ...-.. .. .. (for example "**DAU-GB1**")

spacer type: **A** = -CO_2 spacer (Ch.1) or spacer expulsion by 1,4- or 1,6-elimination (Ch. 4)
B = spacer expulsion by cyclization (Ch.3)

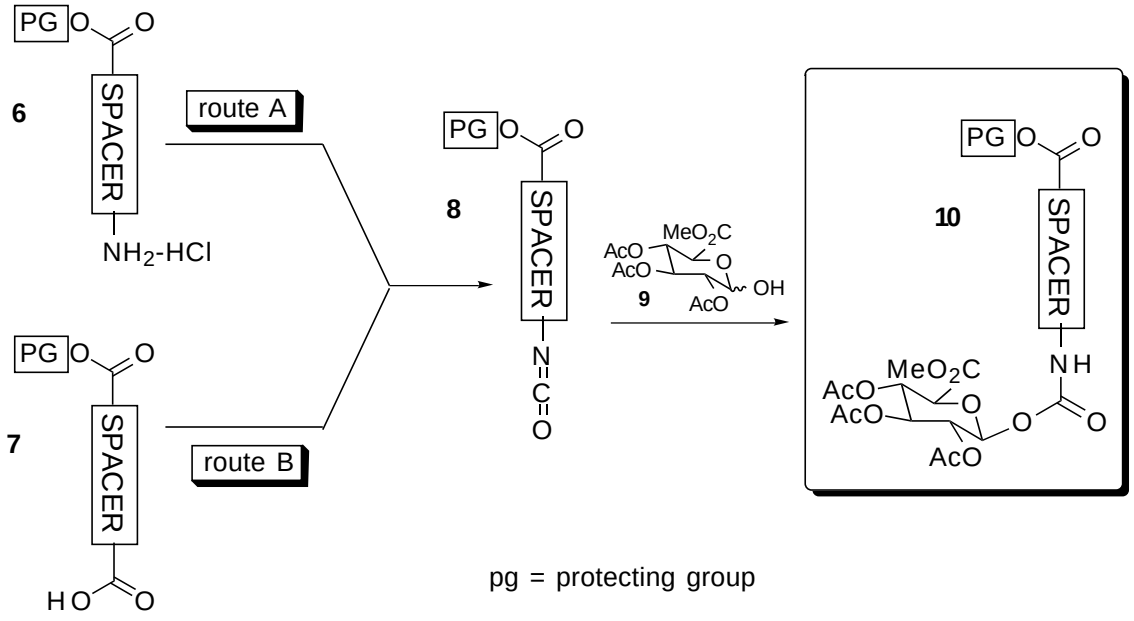
spacer number: numbering of prodrugs of the same type

Chart 3.1 *Target compounds.*

3.3 Synthesis of daunorubicin-3'-*N*-*[spacer]*-*O*-*β*-D-glucuronyl carbamates

3.3.1 Strategy of the synthesis

Considering the multiple functionalities present in daunorubicin, its ease of oxidation and especially the vulnerability of the glycosidic bond connecting the daunorubicinone aglycon and daunosamine sugar (see chart 1.3), the synthesis of target compounds **GB1-6** requires a subtle approach. Preferably, daunorubicin is involved in the synthesis sequence in one of the final reaction steps. For the preparation of a large number of target compounds by merely variation of the starting material, a versatile synthesis with a broad scope is needed.

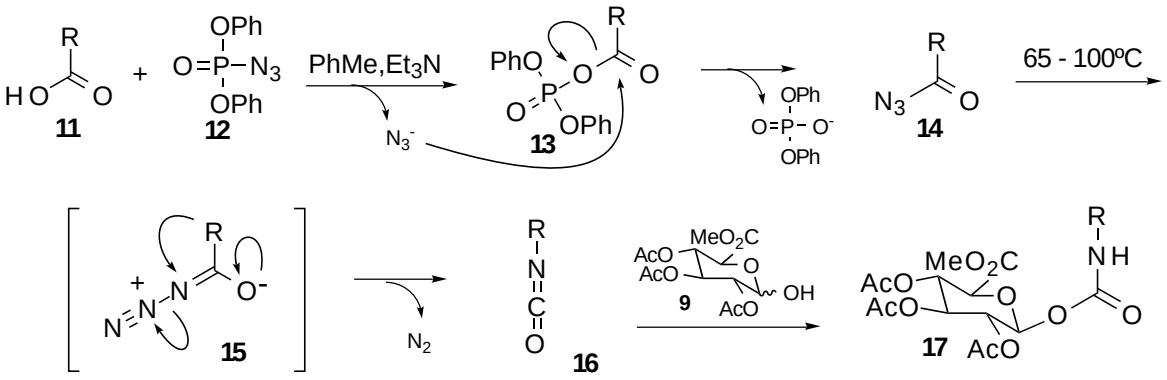


Scheme 3.4 *General synthetic pathways leading to pro-moiety **10**.*

Key compound **10** can be prepared *via* routes A and B (scheme 3.4) by the introduction of a *β*-D-glucuronyl carbamate group to the spacer *via* the addition of anomerically unprotected glucuronic acid **9** to spacer isocyanate **8**. As outlined in section 5.1, this isocyanate addition reaction generally leads to very high *β*-diastereoselectivities and was elaborated during our studies toward the synthesis of *β*-D-glucuronyl carbamate based prodrugs [10]. At first glance, easy access to a large number of prodrugs can be attained using route A in which spacer isocyanate **8** is formed from spacer amine **6**. Because of the desired lactamization potential, however, a serious drawback of the use of γ- and δ-amino esters **6** is their tendency to cyclize prematurely, expelling the protecting group (pg) on the carboxylic acid function. Moreover, only a few γ- and δ-amino acids from which **8** can be prepared are commercially available. Nevertheless, initially prodrug **DAU-GB1** was prepared starting from a γ-amino butyric acid ester following route A.

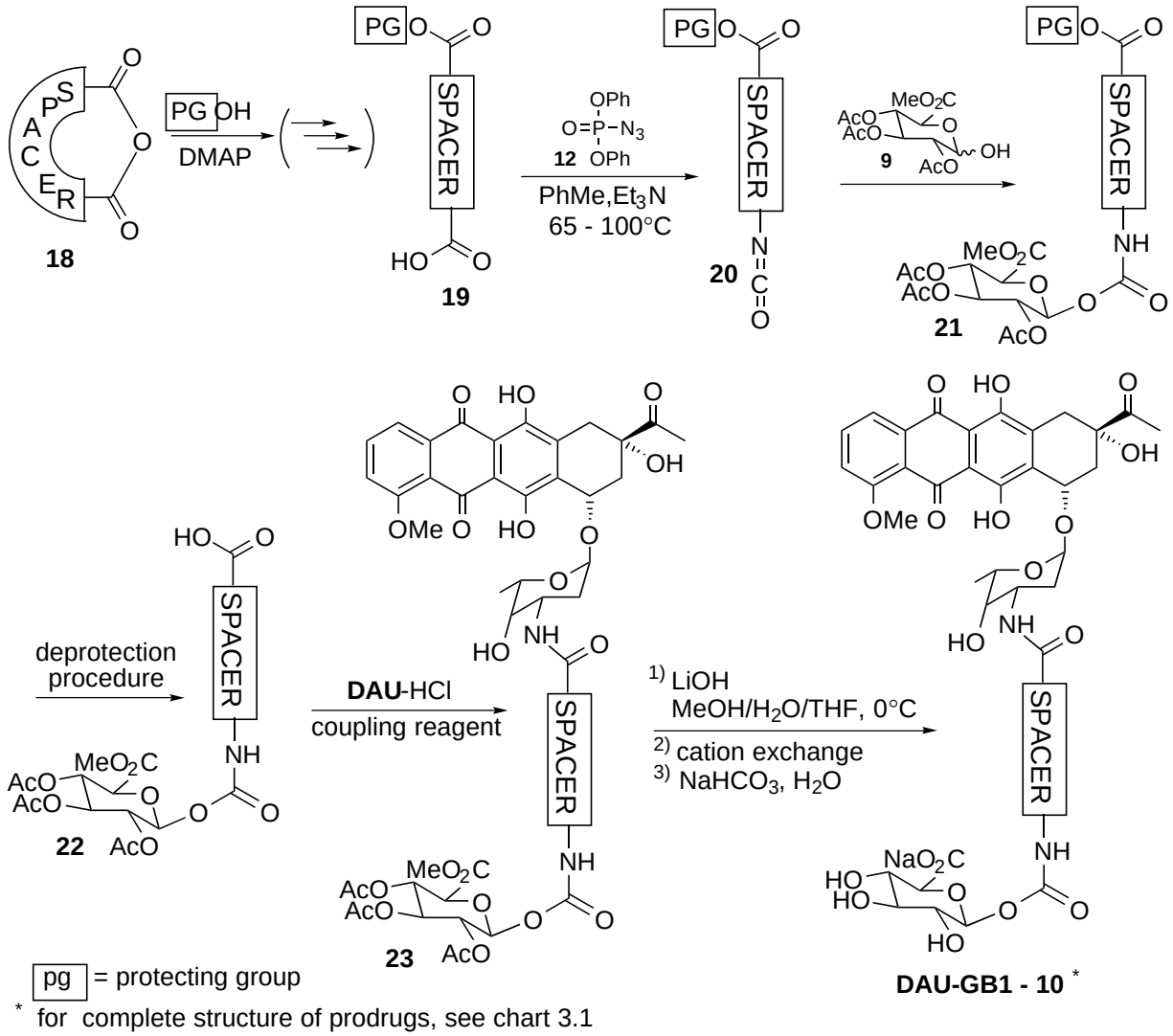
Route B, wherein the synthesis starts from spacer γ- and δ-dicarboxylic acid monoesters **7** offers a number of advantages over route A. The *β*-D-glucuronyl carbamate fragment

can be introduced very conveniently without untimely cyclization of the spacer making use of the Curtius rearrangement to transform a carboxylic acid group into an isocyanate group (scheme 3.5).



Scheme 3.5 *Modified Curtius reaction.*

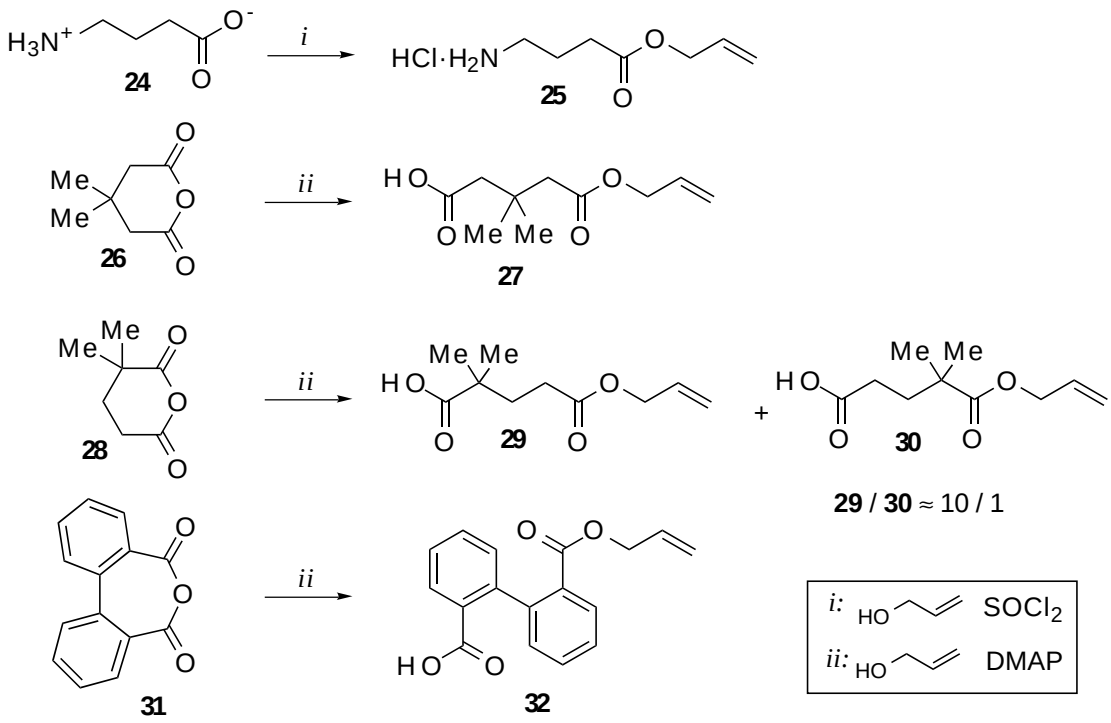
The rearrangement of acyl azides to isocyanates can be employed in the synthesis of prodrugs to generate isocyanates **16** from carboxylic acids **11**. Using diphenylphosphoryl azide **12** [11], spacer acyl azides **14** [12] were formed from carboxylic acids **11** *via* mixed phosphorus anhydrides **13** [13]. Upon heating acyl azides **14** rearranged to isocyanates **16** according to the mechanism depicted in **15**. Diphenyl phosphoryl azide **12** appeared an invaluable reagent for the convenient one-pot preparation of acyl azides directly from carboxylic acids. After the isocyanates had been formed, the mixture was cooled to ambient temperature and anomerically unprotected glucuronate **9** was added. Following this scheme, *β*-D-glucuronyl carbamates **17** can be obtained in a one-pot procedure from acids **11** in good to excellent yields in more than 95% *β*-diastereoselectivity. For further details concerning the addition reaction of **9** and other anomerically unprotected glycosyl donors to isocyanates, see section 5.1.
When applied to the preparation of prodrugs, dicarboxylic acid monoesters **19** (scheme 3.6) are processed in the modified Curtius reaction. These acids **19** are readily accessible from a variety of commercially available γ- and δ-anhydrides **18** and dicarboxylic acids so that the synthetic procedure depicted in scheme 3.6, is a versatile method giving access to a large number of target compounds.



Scheme 3.6 *Synthesis of prodrugs* ***GB1 - 10****.*

3.3.2 Preparation of dicarboxylic acid monoesters

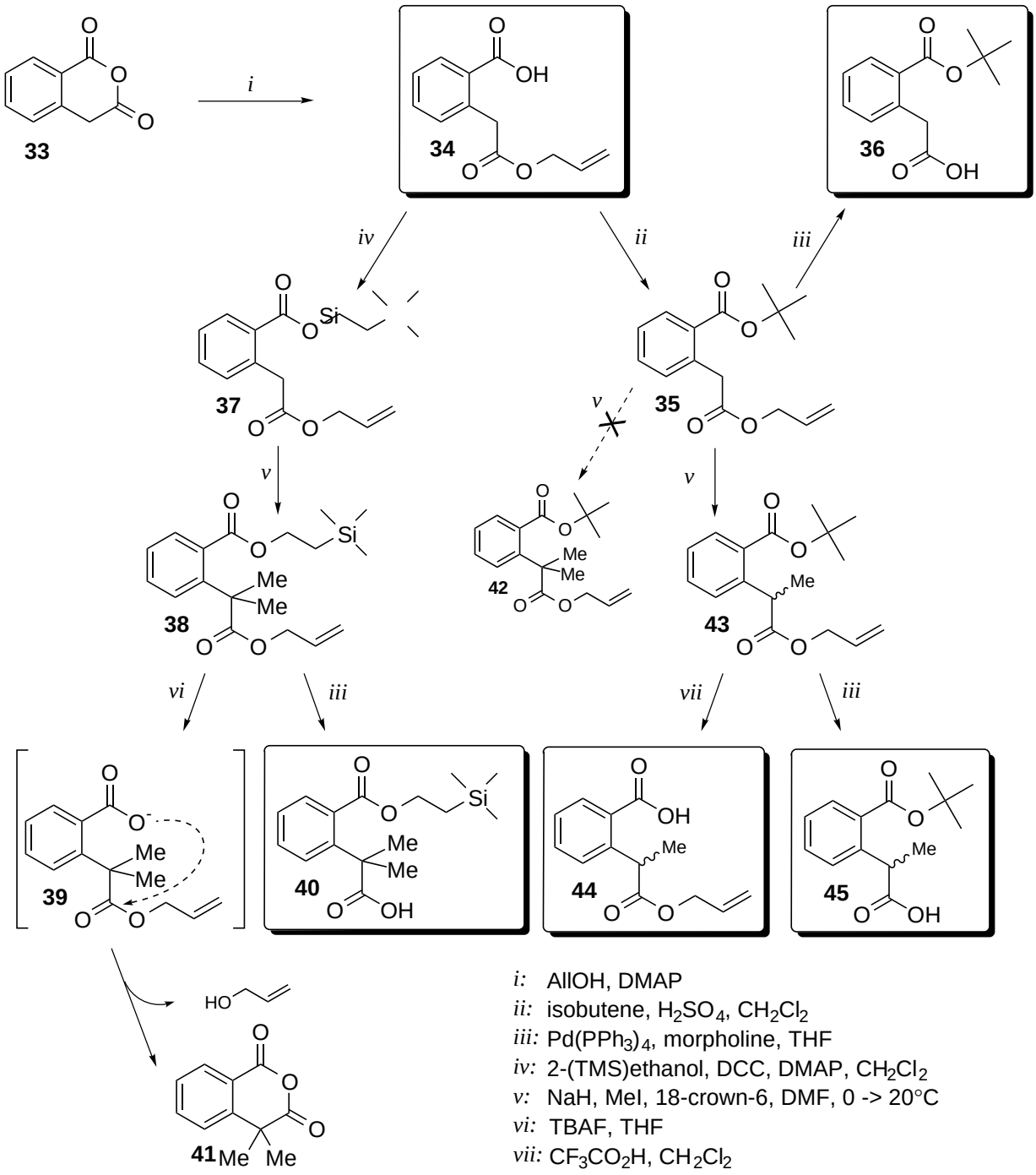
Carboxylic acid esters **19** (scheme 3.6) for prodrugs **DAU-GB2 - 4** are readily prepared by ring opening of the respective commercially available cyclic anhydrides **18**. In case of the unsymmetrical anhydride **28** (scheme 3.7), ring opening resulted in the predominant formation of γ-dimethyl glutaric acid allyl esters **29**. The α-dimethyl isomer **30** was formed to a much smaller extent. It was not possible to determined the ratio of the isomeric products using ^{1}H-NMR because of overlap of signals [14]. The mixture could not be separated and was used as such in subsequent reaction steps. Later on in the synthesis of **DAU-GB3**, however, when the corresponding pro-moieties **48b**/**49b** were coupled to daunorubicin, both isomeric coupling products **48c** and **49c** (scheme 3.9) could be separated.



Scheme 3.7 *Preparation of starting material for prodrugs DAU-GB1-4.*

Homophthalic anhydride **33** (scheme 3.8) is a versatile starting material for the synthesis of prodrugs **DAU-GB5 - 10** (presented in chart 3.1). This unsymmetrical anhydride was opened regioselectively with allyl alcohol affording monoallyl ester **34**, which is the starting material for prodrug **DAU-GB5** (chart 3.1), in a high yield. Because of its efficient and smooth removal, the allyl group was chosen to protect one of the carboxylic acid units of the spacer moiety. Kunz and co-workers have developed a number of methods to unmask allyl-type protected alcohol- amine-, and carboxylic acid functional groups [15]. This methodology is suitable for syntheses in the field of carbohydrate chemistry. The other isomer having the allyl group on the aromatic acid was not detected in this reaction. The preference for opening of the anhydride at the phenylacetic acid side of the molecule is attributed to the facts that the benzoic acid fragment is a better leaving group than the phenylacetic acid part and that in case of attack of a nucleophile on the aromatic acid carbonyl, this would lead to a tetrahedral intermediate in which the conjugation of the carbonyl with the aromatic ring is no longer present.

The starting material for **DAU-GB8** (chart 3.1), *tert*.-butyl ester **36**, was easily prepared from **34** *via* diester **35** (scheme 3.8). Examples of selectively demasking a *tert*.-butyl esters in the presence of carbohydrate acetal functionalities are described in the literature [16].



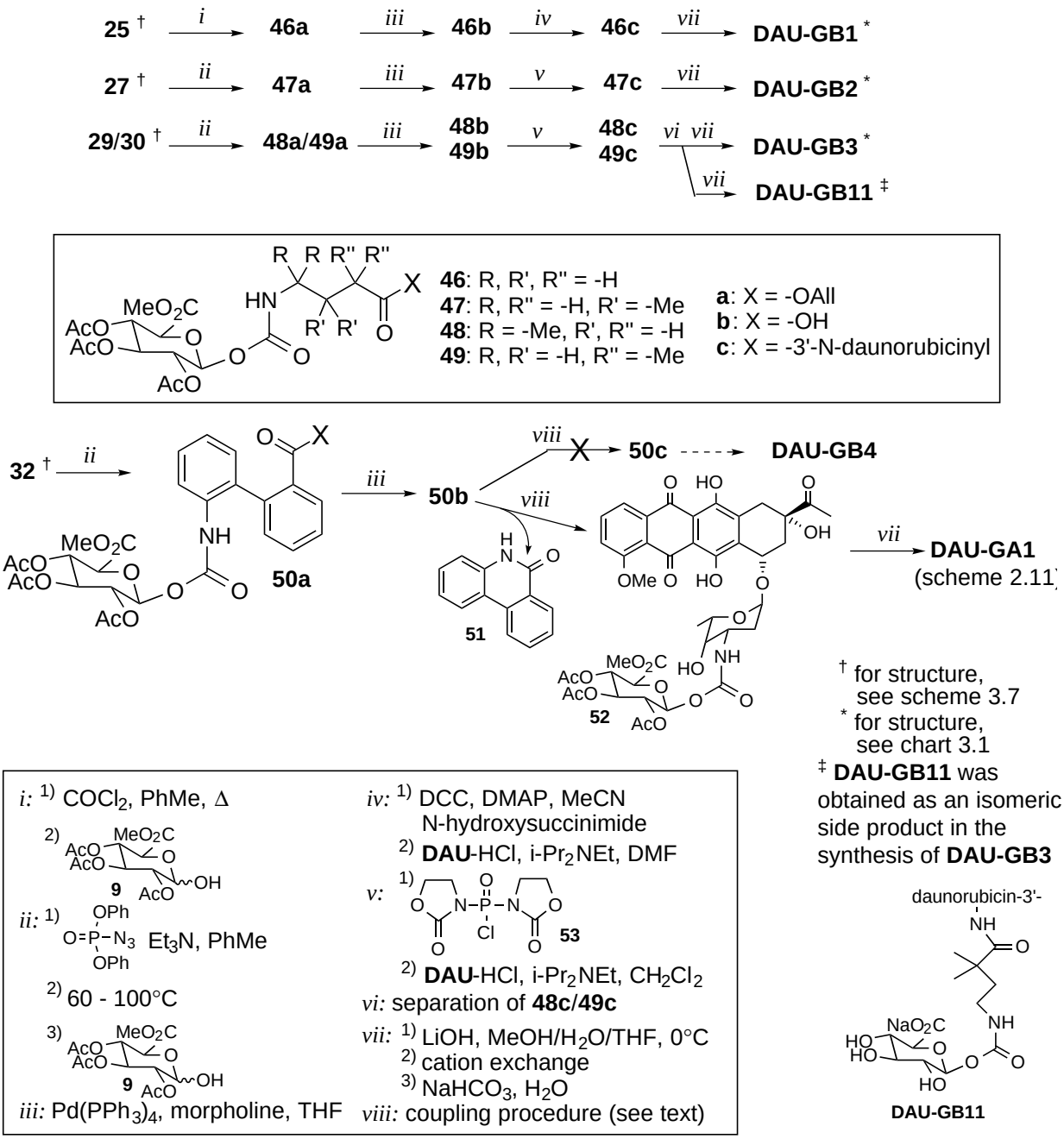
Scheme 3.8 *Preparation of starting material for prodrugs* ***DAU-GB5 - 10****.*

For the synthesis of prodrugs containing additional methyl groups in the spacer, bis-protected homophthalic acid **35** was methylated at the benzylic position using sodium hydride and methyl iodide. Despite variation of reaction temperature and -time and addition of a crown ether, the dimethylated product **42** could not be obtained. Probably due to steric hindrance of the bulky *tert*.-butyl group, only the monomethyl derivative **43** was obtained in all cases. Removal of the *tert*.-butyl group of **43** afforded **44**, being the starting material for prodrug **DAU-GB6**. Removal of the allyl group of **43** led to **45** which is the starting material for **DAU-GB9**.

To study whether the *tert.*-butyl group actually impeded bis-methylation of the benzylic carbon atom of **35**, the carboxylic acid group of **34** was protected with a sterically less demanding group. For this purpose, the 2-trimethylsilylethyl ester **37** was prepared. This functionalized ethyl ester can be unmasked selectively with fluoride ions in the presence of other esters groups in a *β*-elimination reaction to give ethylene, trimethylsilyl fluoride and the carboxylate salt [17]. Furthermore, the ester is unaffected under the reaction conditions required for methylation. In contrast to **35**, bismethylation of **37** was feasable and led to **38**. Removal of the 2-trimethylsilyl ethyl group with TBAF, however, did not lead to the desired product **39** (starting material for **DAU-GB7**), but instead anhydride **41** was obtained instead almost quantitatively. A likely mechanism for this reaction is cyclization by intramolecular attack of the carboxylate anion on the adjacent allyl ester whereby both methyl groups facilitate ring closure by pushing the phenylacetic acid ester towards the benzoic acid fragment (stereopopulation control). Attempts to open *α,α*-dimethyl homophthalic anhydride **41** with allylalcohol analogously to the unsubstituted homophthalic acid **33** failed. Removal of the allyl group of **38** led to **40**, the starting dicarboxylic acid monoester for the synthesis of prodrug **DAU-GB10**.

3.3.3 One-pot synthesis of *β*-D-glucuronyl carbamate pro-moieties from spacer carboxylic acids and further reactions to prodrugs

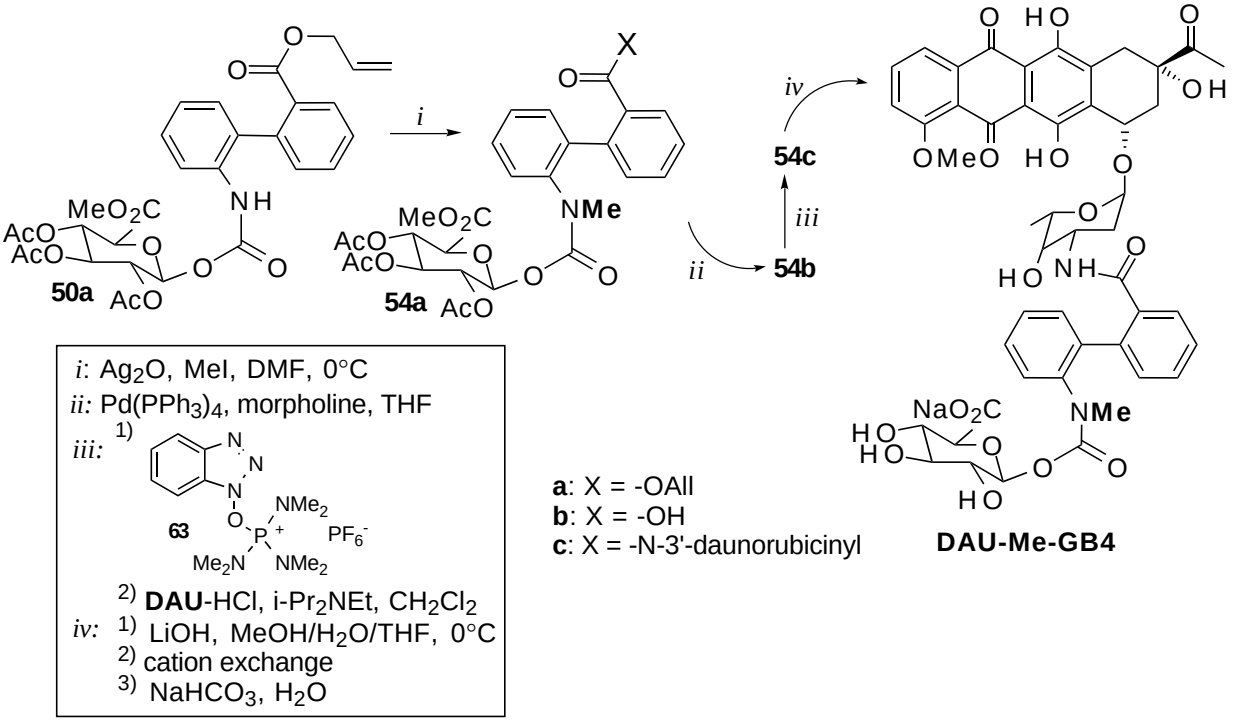
As outlined in scheme 3.6, a one-pot modified Curtius reaction of dicarboxylic acid monoesters with general structure **19** and the subsequent reaction with glucuronyl donor **9** led to protected pro-moieties of general structure **21**. In the specific cases (scheme 3.9), glutaric acid derivatives **27** and the mixture **29**/**30** cleanly gave protected pro-moieties **47a** and **48a**/**49a**, respectively. Protected pro-moiety **46a** was obtained by the addition reaction of glucuronyl donor **9** to the isocyanate obtained from amine hydrochloride **25** and phosgene. The allyl-protected carboxylic acid groups of **46a - 50a** are demasked almost quantitatively using Pd(0) to transfer the allyl group to morpholine [15] that is used in five-fold excess, yielding **46b - 50b**. To obtain the protected prodrugs **46c - 49c**, the carboxylic acid groups of the corresponding pro-moieties **46b - 49b** were coupled to daunorubicin. A number of different methods and reagents were examined for this amide coupling reaction such as DCC, DCC/*N*-hydroxy succinimide, *N,N'*-disuccinimidyl carbonate (DSC) [18], *N,N*-bis[2-oxo-3-oxazolidinyl]phosphorodiamidic chloride (BOP-Cl, compound **53** scheme 3.9) [19], *O*-(benzotriazol-1-yl)-*N,N,N',N'*-tetramethyl uronium tetrafluoroborate (TBTU) [20] and diphenyl phosphoryl azide (DPPA) [11a]. The (benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate reagent [21] (BOP, compound **63** scheme 3.11), however, gave the best results. Analogously to the final deprotection step in the synthesis of **DAU-GA1** (scheme 2.11), target compounds **DAU-GB1 - 3** were obtained after LiOH-mediated hydrolysis of the four protective groups of the *β*-D-glucuronyl entity. Additionally, **DAU-GB11** was obtained after deprotection of the isomeric side product **49c** obtained in the synthesis of **DAU-GB3**.



Scheme 3.9 *Preparation of prodrugs* ***DAU-GB1 - 3****.*

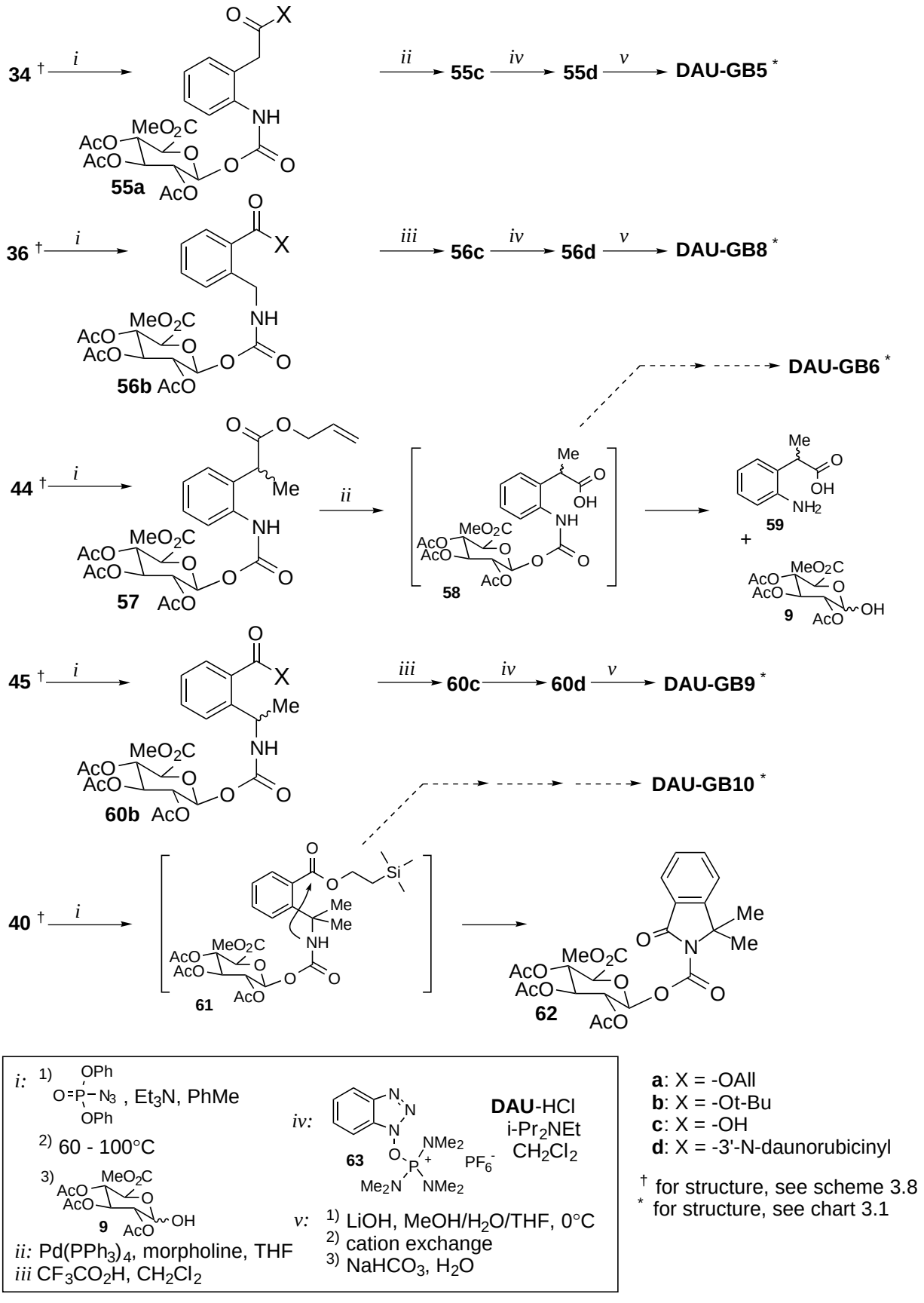
Surprisingly, when it was attempted to couple **50b** to daunorubicin using the BOP coupling reagent (compound **63** scheme 3.10), the expected protected prodrug **50c** containing the biphenyl spacer was not obtained (scheme 3.9). Variation of reaction temperature and use of other peptide coupling reagents for this condensation reaction always produced the protected precursor of **DAU-GA1**, compound **52** (i.e. **38***β* in scheme 2.11) in high yield. Together with this compound, the cyclized biphenyl spacer, viz phenanthridinone **51**, was formed. Apparently, cyclization of the biphenyl spacer is very facile and target prodrug **DAU-GB4** was not obtained because of this premature spacer elimination. It is of interest to note that this novel glycosyl-carbonyl transfer reaction offers the alternative possibility to prepare *β*-D-glycosyl carbamates from

primary or secondary amines which can not be transformed into isocyanates to make β-D-glycosyl carbamates. This new chemistry is further outlined in section 5.2.
To avoid premature cyclization of the **-GB4** spacer, methylation of the spacer nitrogen atom was considered to prevent intramolecular attack of this nitrogen atom on the adjacent ester (for a mechanism of the cyclization reaction, see section 5.2, scheme 5.7). When the *N*-biphenyl *O*-β-D-glucuronyl carbamate **50a** was treated with silver oxide and methyl iodide in DMF [22] (scheme 3.10), the nitrogen atom was methylated and yielded the *N*-methyl derivative **54a**. Removal of the allyl group using Pd(0) / morpholine and subsequent coupling of the resulting acid **54b** to daunorubicin using BOP, resulted in the desired coupling product **54c**. Using the *N*-methyl analog of the **-GB4** pro-moiety to prepare the corresponding prodrug, no spacer elimination took place as was the case for the conversion of **50b** into **52** (in scheme 3.9). Deprotection of **54c** resulted in the *N*-methylated prodrug **DAU-Me-GB4**.



Scheme 3.10 *Synthesis of N-methyl* ***DAU-GB4****.*

Analogously to the syntheses of prodrugs **DAU-GB1** -**3** (scheme 3.9), homophthalic acid monoesters **34**, **36** and **45** (depicted in scheme 3.8) were processed to obtain prodrugs **DAU-GB5**, **-GB8** and **-GB9**, respectively (scheme 3.11). Using trifluoroacetic acid and carefully excluding moisture, the *tert.*-butyl group of **56b** and **60b** was easily removed whilst the carbohydrate acetal linkage was left unaffected[16]. When demasking the allyl ester of **57** (obtained from mono α-methylated acid **44**, depicted in scheme 3.8), only glucuronic acid **9** and amino acid **59** were isolated. The fragmentation of free carboxylic acid **58** is probably due to acid-catalyzed hydrolysis of the β-D-glucuronyl carbamate by the carboxylic acid during work-up. Similarly to this, demasking of allyl ester **55a** to **55c** led to decomposition products, but sufficient intact pro-moiety was isolated and could be coupled to daunorubicin [23].



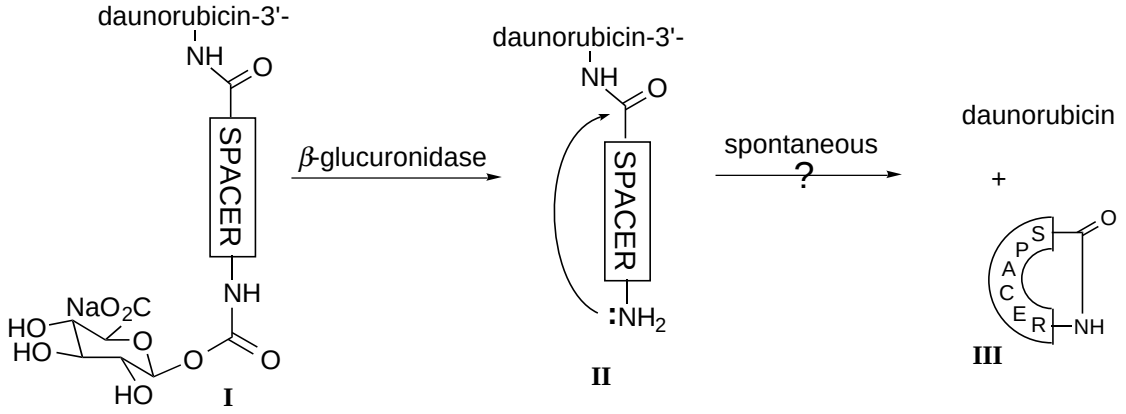
Scheme 3.11 *Preparation of prodrugs* ***DAU-GB5, -GB8*** *and* ***-GB9.***

When α,α-dimethyl homophthalic acid derivative **40** was processed in the modified Curtius reaction following the usual procedure (see general procedure #2 in the experimental section), no reaction was observed. When the reaction mixture was heated to 95°C after glucuronyl donor **9** had been added, exclusively cyclization product **62** was obtained in 39% yield after column chromatography as was established by ^{1}H-NMR and mass-spectrometry. This premature cyclization reaction demonstrates that the geminal methyl groups on the spacer indeed facilitate the intramolecular cyclization.

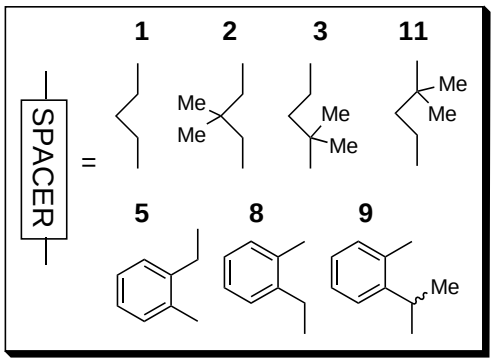
3.4 Evaluation of the synthesized prodrugs for application in ADEPT [24]

3.4.1 Enzymatic activation rates

The synthesized prodrugs were designed to be activated to daunorubicin in a two step process, according to scheme 3.12, viz. step *i.* Hydrolysis of the β-D-glucuronyl carbamate specifier by β-glucuronidase and step *ii.* self elimination of the spacer by ring closure. Spacers were incorporated between drug and specifier to facilitate enzymatic hydrolysis.



DAU-GB1 - 3, **5**, **8**, **9** and **11**



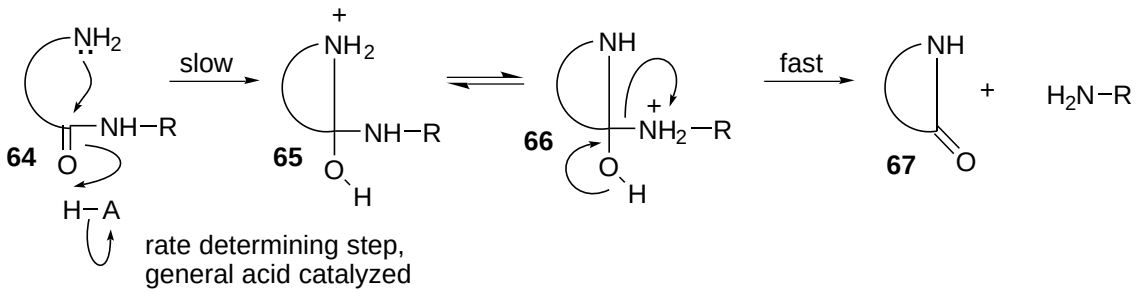
Scheme 3.12 *Hydrolysis of prodrugs.*

Prodrug	Hydrolysis $t_{1/2}$
DAU-GB1 [24]	3000
DAU-GB2	52
DAU-GB3	130
DAU-Me-GB4 *	>5000 †
DAU-GB5 [24]	36
DAU-GB8	5
DAU-GB9	>1000
DAU-GB11	570

100 μM prodrug, 1 μg/mL enzyme, pH = 6.8, 37°C.
* For structure, see scheme 3.10.
† Activation to daunorubicin.

Table 3.2 *β-Glucuronidase mediated hydrolysis of prodrugs to drug-spacer molecules.*

The structure of the spacer influences both step *i.* and *ii.*. Because detailed information concerning the active site of human *β*-glucuronidase is not known, construction of a spacer to achieve optimal hydrolysis rates on rational grounds is not possible. In contrast to this "black box" situation of step *i.* (scheme 3.12), the cyclization reaction of step 2 is much better understood. As described in section 3.2.2, the intramolecular cyclization reaction is accelerated by stereopopulation control of the spacer. Under physiological conditions, a proper preorganization of the spacer is a prerequisite for a favorable elimination rate. Therefore, prodrugs were designed containing spacers with increasing cyclization tendency according to stereopopulation control. Disappointingly, the synthesis of three of the designed prodrugs, **DAU-GB6**, **7** and **10** (see chart 3.1) could not be accomplished. Prodrugs **DAU-GB7** and -**10** were expected to have high cyclization rates on account of stereopopulation control.
Concerning the mechanism of spacer elimination, the rate-determining step in the intramolecular aminolysis of amides is a general-acid-catalyzed formation of tetrahedral intermediate **65** (scheme 3.13) [5a, 7d, 8a]. The electronic nature of the leaving amine was reported to have a minor effect on the rate constant because breakdown of the tetrahedral intermediate **66** occurs after the rate-determining step [5a, 7d, 8a]. Therefore, in case of the present prodrugs, it was expected that the poor leaving ability of 3'-*N*-daunorubicinyl group would not impede the intramolecular cyclization reaction.



Scheme 3.13 *Cyclization of amino amides.*

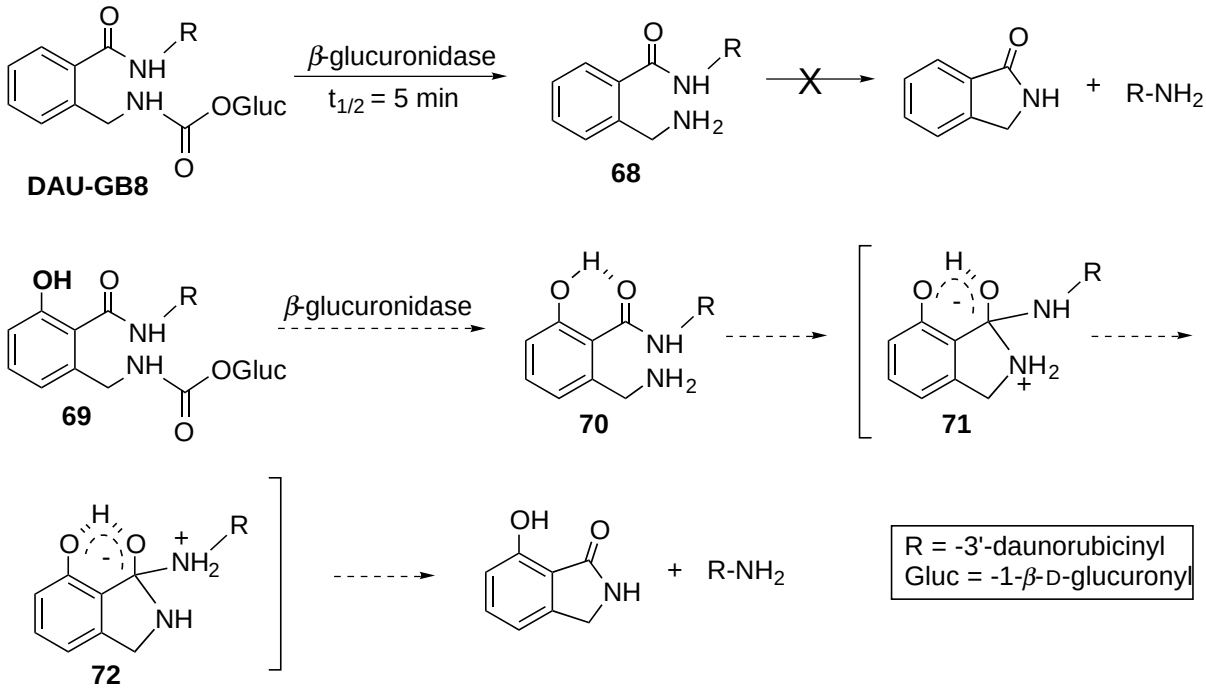
For the determination of the enzymatic activation rates, prodrugs were incubated with human *β*-glucuronidase at pH = 6.8 [25]. Compared to **DAU-GA1** (Ch. 2), which was activated very slowly by *β*-glucuronidase ($t_{1/2}$ = 8200 min, see table 2.3), all the present prodrugs were hydrolyzed more easily (table 3.2). The incorporation of a spacer between drug and specifier proved to accelerate enzyme hydrolysis rates. Disappointingly, all prodrugs, except **DAU-Me-GB4**, were not converted to daunorubicin upon hydrolysis by *β*-glucuronidase but to the corresponding drug-spacer molecule (scheme 3.12). The desired elimination of the spacer by ring closure did not take place and daunorubicin was not recovered. When **DAU-Me-GB4**, was incubated with *β*-glucuronidase, daunorubicin was formed but the activation half-life was too slow to be of interest for application in ADEPT ($t_{1/2}$ >5000 min).
although the present prodrugs were not activated to produce daunorubicin, the rates of the enzymatic hydrolysis of some prodrugs were remarkable. Compound **DAU-GB8** was hydrolyzed smoothly (see table 3.2), about 1600 times faster than **DAU-GA1**. A

striking difference in hydrolysis rates was observed for prodrugs containing additional methyl groups on the aliphatics spacer when compared to **DAU-GB1**. Hydrolysis rates of these prodrugs (**DAU-GB2**, **-GB3** and **-GB11**) were increased. On the other hand, prodrug **DAU-GB9**, having an additional methyl group on the benzylic position of the spacer compared to **DAU-GB8**, was hydrolyzed very slowly ($t_{1/2}$ >1000 min), also spacer elimination did not take place. In this case the additional methyl group retarded the hydrolysis more than 200-fold (table 3.2). In this light, prodrug **DAU-GB10** (its synthesis could not be accoplished) containing *two* additional methyl groups compared to **DAU-GB8** (chart 3.1), is expected to be hydrolyzed by *β*-glucuronidase very slowly. It is therefore concluded that stereopopulation control does not seem a conceivable strategy to realize immolative spacer cyclization, because stereopopulation control has an opposing effect on the initial enzymatic hydrolysis step.

3.4.2 Cytotoxicities

The *in vitro* cytotoxicities of all prodrugs [24] were determined on OVCAR-3 cells and compared to the toxicity of daunorubicin. the prodrugs were 20 to 100-fold less toxic than daunorubicin. Because the prodrugs of the present chapter were not activated to produce daunorubicin, exact data of prodrug cytotoxicities were not collected.

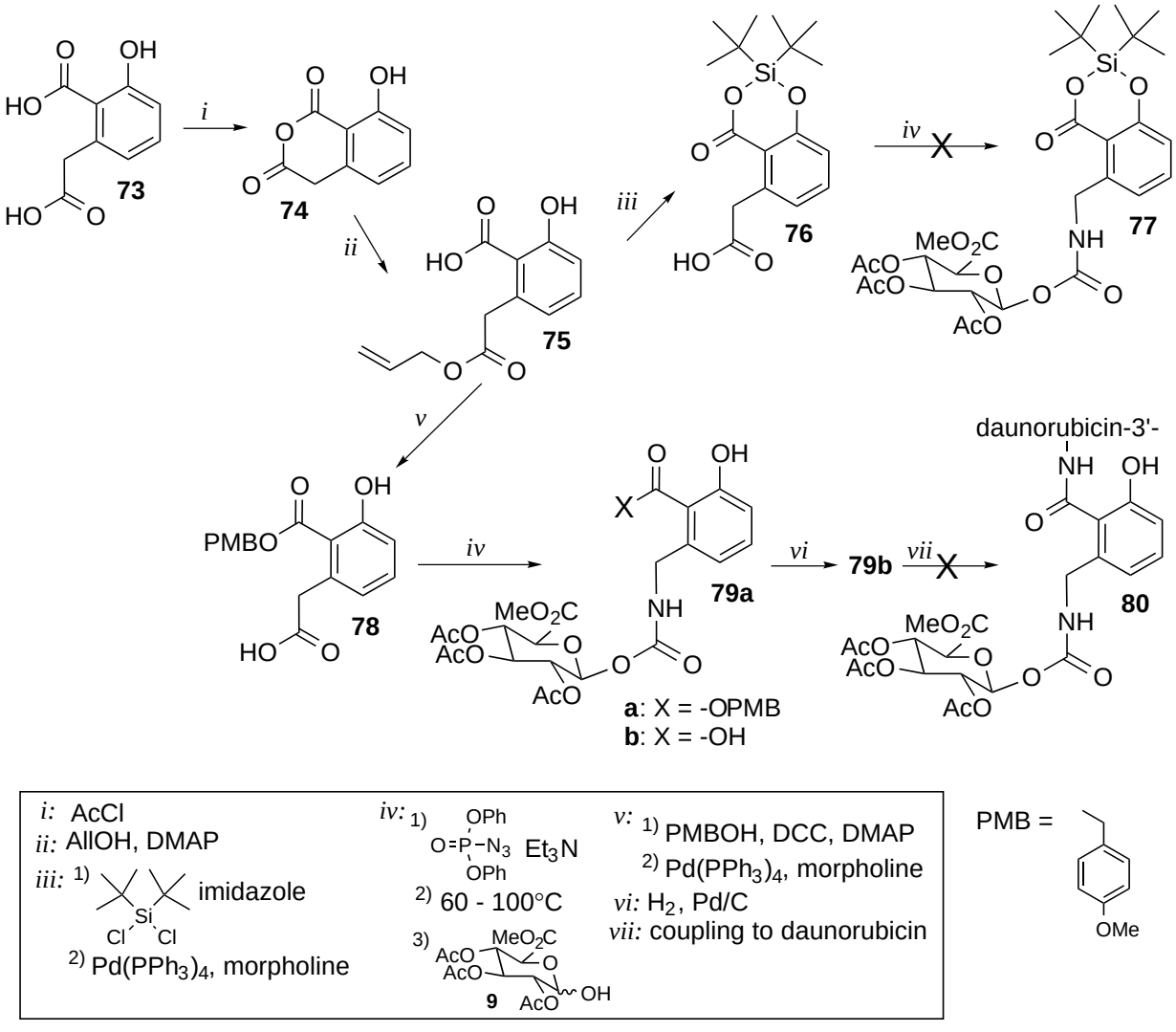
3.5 Alternative approach to spacer cycloelimination



Scheme 3.14 *Intramolecularly catalyzed spacer cyclization.*

In scheme 3.14, a possibility is presented to accomplish spacer elimination of prodrug **DAU-GB8**, which was hydrolyzed very fast by *β*-glucuronidase to the corresponding drug-spacer molecule ($t_{1/2}$ = 5 min, scheme 3.12 and table 3.2). As mentioned earlier, drug-spacer molecule **68**, obtained from **DAU-GB8** after *β*-glucuronide hydrolysis, did

not convert to daunorubicin and the corresponding lactam. The inability of **68** to undergo spontaneous cycloelimination may be attributed to a low reactivity of the amide function in this substrate to nucleophylic attack. It is now proposed to stabilize the initial sp^3 intermediate during the intramolecular aminolysis through anchimeric assistance. This can, in principle, be realized by the introduction of a hydroxyl group in the *ortho*-position to the amide carboxyl function, as in **69** (scheme 3.14). The intramolecular aminolysis should then proceed *via* the stabilized sp^3 intermediates **71** and **72** to eventually give cycloelimination as shown in scheme 3.14. In the literature, the proposed anchimeric assistance of an *ortho*-hydroxy group has been demonstrated for the intramolecular aminolysis of an ester, whereby this hydroxyl function served as an intramolecular general acid catalyst [26].



Scheme 3.15 *Attempts to prepare prodrug **69** (scheme 3.14).*

Disappointingly, however, attempts to prepare prodrug **69** failed (scheme 3.15). For the purpose outlied above, 6-hydroxy homophthalic acid (**73**), prepared according to literature procedures [27], was converted to the anhydride **74** which was opened with allylalcohol to give allylester **75**. This compound was *bis*-protected with the di-*tert*.-

butylsilene group and the allyl ester was removed to give compound **76**. A modified Curtius reaction of **76**, however, did not give the desired *β*-D-glucuronyl carbamate product **77**. Loss of the silyl protecting group and subsequent side product formation was observed. It was then decided to prepare prodrug **69** from acid **73** analogous to the synthesis of **DAU-GB8** from **36** (scheme 3.8 and 3.11) but using a *p*-methoxybenzoyl protecting group instead of a *tert.*-butyl protecting group. Modified Curtius reaction of **78** led to the *β*-D-glucuronyl carbamate **79a**. After removal of the *p*-methoxybenzyl group of **79a**, however, it was not possible to couple the resulting acid **79b** to daunorubicin using the BOP coupling reagent and as a consequence compound **80** was not obtained. Other coupling reactions have sofar not been tried. In spite of the disappointing results, compound **69** remains an interesting candidate as a prodrug.

3.6 Experimental part

3.6.1 Biological evaluation

Cytotoxicities

The cytotoxicities of the prodrugs were determined as described in section 4.5.1.

Prodrug activation half-lives

The enzymatic activation rates of the prodrugs were determined by incubation of 100 μM of prodrug in 0.1% BSA/PBS at pH = 6.8 with 0.03 U/mL human *β*-glucuronidase at 37°C. Samples were prepared and analyzed on reversed phase SiO_2-C_{18} HPLC as described [24].

3.6.2 Chemistry

General

Daunorubicin hydrochloride **DAU**-HCl was a generous gift from PharmaChemie BV Haarlem. Chromatotron model 7924-T Harrison Research (Palo Alto, California, USA) equipped with plates (thickness 2 mm, diameter 8.5 cm) made form Merck silicagel 60 PF_{254} which contains gypsum (art. 7749) was used when circular chromatography is indicated. Reversed phase chromatography was performed with a liquid chromatography pump LC-410 (Kontron) using a pre-packed column (24 cm, diameter 11 mm) containing octadecylsilane (40-63 μm) (Merck, Darmstadt). Prior to use the RP-C_{18} column was equilibrated with demineralized water. ^{1}H NMR spectra at 400 MHz were obtained on a Bruker AM-400 spectrometer, spectra at 100 MHz on a Bruker AM-100 spectrometer. Chemical shifts are expressed in ppm downfield from internal standard Me_4Si. All solvents were dried before use: Et_3N, CH_2Cl_2 and PhMe were dried by distillation over CaH_2, pyridine by distillation over $CaCl_2$ and THF by distillation over $LiAlH_4$ or sodium. *i*-Pr_2NEt was dried over KOH pellets. In all cases demineralized H_2O was used. Allyl alcohol was dried by distillation over Mg/I_2.

General Procedure #1: Opening of cyclic anhydrides with AllOH (scheme 3.7 and 3.8).

The commercially available anhydrides were dissolved in AllOH (2.0 g anhydride in 10-15 mL AllOH) and *ca.* 10 mol% DMAP was added and the reaction was stirred overnight. After that, the AllOH was removed by evaporation under reduced pressure and the residue was dissolved in Et_2O and washed

successively with 100 mL portions of 0.5 N aqueous $NaHSO_4$ and brine. The organic fraction was dried over Na_2SO_4 and concentrated to dryness. Traces of allyl alcohol were removed by azeotropic distillation with *n*-Hex to obtain the respective dicarboxylic acid monoesters in > 80% yield as colorless liquids.

General procedure #2: Modified Curtius procedure for conversion of carboxylic acids to *β*-D-glucuronyl carbamates (scheme 3.9 and 3.11).

500 mg of dicarboxylic acid mono ester prepared as described above was stirred overnight with 1.1 equiv. of $(PhO)_2P(O)N_3$ and 1.1 equiv. of Et_3N in 15 mL of dry PhMe under an argon atmosphere at room temperature. The reaction mixture was subsequently heated until gas evolution was observed, occurring between 65-100ºC depending on the actual substrate. heating was continued for 2 h. In case of aromatic or benzylic isocyanates, the reaction mixture was allowed to cool to ambient temperature and for aliphatic isocyanates the reaction mixture was cooled down to 60ºC. 0.5-0.75 equiv. [28] of the anomerically unprotected glucuronate **9** were added. The reaction mixture was stirred until **9** had almost disappeared on TLC (SiO_2, Et_2O). After that, the reaction mixture was diluted with 100 mL of Et_2O and successively washed with 200 mL portions of aqueous 0.5 N $KHSO_4$, saturated aqueous $NaHCO_3$ (3x) and brine. The organic layer was dried over Na_2SO_4 and evaporated. The residual material was purified by column chromatography (SiO_2, Et_2O/*n*-Hex 4/1) to yield the respective 1-*O*-*β*-D-glucuronyl carbamates in good to excellent yield and in 95-100% *β*-diastereoselectivity.

General procedure #3: Demasking of allyl esters using Pd(0) (scheme 3.8 to 3.11).

300 mg of allyl ester was dissolved in 25 mL of dry THF and 5 equiv. of morpholine were added. During 30 min, argon was bubbled through the solution to remove oxygen. After that, a catalytic amount of $Pd(PPh_3)_4$ was added and the mixture was stirred for 20 min under an argon atmosphere. Completion of the deprotection was checked by TLC (SiO_2, Et_2O) and when necessary, an additional small amount of the Pd(0) complex was added. After all starting material had disappeared, 100 mL of an aqueous 5% $KHSO_4$ solution was added. The aqueous solution was washed with two 100 mL portions of Et_2O, the organic extract was washed with brine, dried over Na_2SO_4 and evaporated. To remove the Pd complex, the residue was dissolved in either *n*-Hex, *i*-Pr_2O or Et_2O depending on the polarity of the product, and the precipitated orange-brown Pd complex was filtered of. The solvent was removed to furnish the free carboxylic acid in (near) quantitative yield. ^{1}H-NMR spectra are not given because they are similar to the respective allyl ester precursors except for the absence of the allyl protons. Coupling of the thus obtained pro-moieties to daunorubicin was executed without delay to minimize decomposition of the pro-moiety.

General procedure #4: Demasking of *tert.*-butyl esters using CF_3CO_2H (scheme 3.8 and 3.11).

100 mg of *tert.*-butyl ester was dissolved in 2.0 mL of CH_2Cl_2 under an argon atmosphere and 300 *μ*L of CF_3CO_2H was added. After 1 h, the solvents were evaporated and traces of CF_3CO_2H were removed under high vacuum to yield the free carboxylic acids in near quantitative yield. ^{1}H-NMR spectra are not given because they are similar to the respective *tert.*-butyl ester precursors except for the absence of the *tert.*-butyl protons. Coupling of the thus obtained pro-moieties to daunorubicin was executed without delay to minimize decomposition of the pro-moiety.

General procedure #5: Coupling of the pro-moiety-CO_2H group to the 3'-NH_2 function of daunorubicin using BOP-Cl or BOP (scheme 3.9 and 3.11).

BOP-Cl coupling

100 mg of pro-moiety-CO_2H **47b** or **48b**/**49b** and 2.5 equiv. of *i*-Pr_2NEt were dissolved in CH_2Cl_2 and cooled to 0ºC. 1.2 equiv. of BOP-Cl **53** (scheme 3.9) were added. The mixture was allowed to warm to ambient temperature and was stirred for 1 h and 1.5 equiv. of **DAU**-HCl and 3 equiv. of *i*-Pr_2NEt were added. The coupling reaction was stirred overnight and worked-up as described below.

BOP coupling

100 mg of pro-moiety-CO_2H **55c**, **56c** or **60c**, 1.1 equiv. of BOP **63** (scheme 3.11), 3 equiv. of *i*-Pr_2NEt and 1.1 equiv. of **DAU**-HCl were stirred under an argon atmosphere at ambient temperature for 15-120 min, the course of the coupling reaction was monitored on TLC (SiO_2, CH_2Cl_2/EtOH 10/1). After that, the reaction mixture was diluted with 150 mL of CH_2Cl_2 and washed successively with 200 mL portions of aqueous 0.5 N $KHSO_4$ (3x), saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over

Na_2SO_4 and evaporated. The resulting red residue was purified by means of circular chromatography using a chromatotron supplied with a 2 mm thick silica plate and mixtures of CH_2Cl_2/EtOH 10/1 (first run) and 20/1 or 30/1 (successive runs) until the protected prodrug was a single spot on TLC (SiO_2, CH_2Cl_2/EtOH 20/1). After evaporation of the eluent, the product was sonicated in *i*-Pr_2O and collected by filtration to yield the protected prodrug as red powder.

General procedure #6: Deprotection of the β-D-glucuronyl specifier in the protected prodrugs (scheme 3.9 and 3.11).

To the protected prodrug were added 6 equiv. of a 0.10 N LiOH solution in MeOH/H_2O/THF 2.5/1/0.5. the resulting deep blue solution was stirred at 0ºC under an argon atmosphere and progress of the deprotection reaction was continuously monitored by reversed phase TLC (SiO_2-C_{18}, MeCN/H_2O 1/1). After 15-90 min of deprotection, the reaction mixture was diluted to *ca.* twice the volume with H_2O and neutralized by adding *ca.* 2 g of amberlite cation exchange material (H^+ form) per 100 mg of protected prodrug, 25 vol% of THF were added. This was stirred until the deep blue solution turned red. The amberlite material was removed by filtration and *ca.* 10 equiv. of $NaHCO_3$ were added to convert the glucuronyl carboxylic acid to the sodium salt. The MeOH and THF suspended in the water layer were removed by evaporation and the red aqueous product solution was transferred it to a reversed phase column packed with RP-C_{18} material. To remove the excess of $NaCO_3$, the column was eluted with *ca.* 300 mL of H_2O. The product stayed on the column. The column was then washed with MeCN/H_2O 1/4 to elute the product and the MeCN was removed by evaporation. In cases where the prodrug was not a single spot on TLC, the aqueous solution was again transferred to the reversed phase column and purified once more by eluting a MeCN/H_2O mixture in a ratio of 1/4 to 1/10. The MeCN was removed by evaporation and freeze drying of the aqueous product solution afforded the prodrug as red fluffy solid.

General procedure #7: α-methylation of homophthalic acid diesters 37 and 35 (scheme 3.8).

500 mg of **37** or **35** was dissolved in 10 mL of DMF and 10 equiv. of MeI and 0.1 equiv. of 18-crown-6 were added and the mixture was cooled to 0ºC. 4 equiv. of NaH were added and the reaction mixture was stirred for 3 days at ambient temperature under an argon atmosphere. After that, the mixture was diluted with *ca.* 100 mL of *n*-Hex and washed with 100 mL portions of a 5% aqueous solution of $KHSO_4$, H_2O, saturated aqueous $NaHCO_3$ and brine. The solution was dried over Na_2SO_4 and evaporated. The crude diester was purified by flash-column chromatography (SiO_2, Et_2O/*n*-Hex 1/6) to yield **38** or **43**, respectively, as slightly yellow oils.

Synthesis of *N*-γ-(daunorubicin-3'-*N*-butyryl) *O*-β-D-glucuronyl carbamate sodium salt (DAU-GB1).

γ-Amino butyric acid allyl ester (**25**). 7.5 g of **24** was dissolved in 75 mL of AllOH and cooled to 0ºC. 40 mL (2.9 equiv.) of $SOCl_2$ were added slowly using a dropping funnel. The reaction was stirred overnight at ambient temperature. After this, the reaction mixture was filtered to remove solid material and the filtrate was evaporated. AllOH was removed by azeotropic distillation with *n*-Hex. The 13.7 g thus obtained brown oil of impure **25** was used without further purification.

γ-Isocyanato butyric acid allyl ester. 13.7 g of impure **25** was dissolved in 75 mL of PhMe. During 2.5 h, the solution was heated to reflux while bubbling phosgene through. After this, argon was bubbled through and stirring was continued for 1 h. The reaction mixture was concentrated by evaporation under reduced pressure and distilled to give the isocyanate, 8.2 g 67% from **24**, as a colorless oil, bp 82ºC (2 mm Hg). ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.93 (k, 2H, -$CH_2CH_2CH_2$-, *J* = 6.5 Hz), 2.46 (t, 2H, -$CH_2CH_2CO_2$-, *J* = 7.0 Hz), 3.41 (t, 2H, -CH_2CH_2NCO, *J* = 6.5 Hz), 4.59 (dt, 2H, -OCH_2CH=, *J* = 1.2 Hz *J* = 5.6 Hz), 5.15-5.45 (m, 1H, =CH_2), 5.70-6.15 (m, 1H, -CH=).

N-γ-(Butyric acid allyl ester) O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**46a**). 63 μL Of the latter isocyanate was dissolved in 5 mL of PhMe and 104 mg (0.8 equiv.) of **9** and a catalytic amount of Et_3N were added. The reaction was stirred overnight at 50ºC and worked up as described according to general

procedure #2 to obtain 139 mg of **46a** in 74% yield and 95% β-diastereoselectivity as a colorless oil, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.70-1.90 (m, 2H, -$CH_2CH_2CH_2$-), 2.01 (s, 9H, 3 -OAc), 2.36 (t, 2H, -$CH_2CH_2CO_2$-, *J* = 7.4 Hz), 3.20 (dd, 2H, -CH_2CH_2NH-, *J* = 6.7 Hz *J* = 12.9 Hz), 3.69 (s, 3H, -CO_2Me), 4.17 (d, 1H, Gluc5-H, *J* = 9.3 Hz), 4.54 (dt, 2H, -OCH_2CH=, *J* = 1.4 Hz *J* = 5.6 Hz), 5.05-6.10 (m, 6H, =CH_2 -CH= -NH- Gluc2,3,4-H), 5.69 (d, 1H, Gluc1-H, *J* = 7.6 Hz).

N-γ-(Daunorubicin-3'-N-butyryl) O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**46c**). The allyl ester of **46a** was removed according to general procedure #3 furnishing **46b** which was coupled to daunorubicin without delay. 70 mg (0.172 mmol) of **46b** and 24 mg (1.2 equiv.) of *N*-hydroxy succinimide were dissolved in 11 mL of MeCN and cooled to 0ºC. 37 mg (1.05 equiv.) of DCC and a catalytic amount of DMAP were added. The reaction was stirred at 0ºC for 1 h and additionally for 1 h at ambient temperature. After this the mixture was filtered and evaporated. The crude active ester thus obtained was dissolved in 10 mL of DMF and 104 mg (1.05 equiv.) of **DAU**-HCl and 77 μL (2.5 equiv.) of *i*-Pr_2NEt dissolved in 5 mL of DMF were added. The reaction was stirred overnight and worked-up according to general procedure #5 to obtain **46c** in 71%, mp 90-95ºC, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.30 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.65-2.25 (m, 6H, 2'$_{ax}$-H 2'$_{eq}$-H 8$_{ax}$-H -$CH_2CH_2CH_2$- 4'-OH), 2.02, 2.03 and 2.04 (3s, 9H, 3 -OAc), 2.32 (d, 1H, 8$_{eq}$-H, *J* = 14.7 Hz), 2.42 (s, 3H, 9-C(O)Me), 2.80-3.00 (m, 2H, -CH_2CH_2C(O)-), 2.88 (d, 1H, 10$_{ax}$-H, *J* = 18.8 Hz), 3.10-3.35 (m, 2H, -CH_2CH_2NH-), 3.20 (d, 1H, 10$_{eq}$-H, *J* = 18.6 Hz), 3.69 (s, 1H, 4'-H), 3.74 (s, 3H, C(O)OMe), 4.05 (s, 3H, 4-OMe), 4.10-4.25 (m, 3H,Gluc5-H 5'-H 3'-H), 4.52 (s, 1H, 9-OH), 5.10 (t, 1H, Gluc2-H, *J* = 8.5 Hz), 5.15-5.40 (m, 3H, Gluc3,4-H 7-H), 5.49 (d, 1H, 1'-H, *J* = 3.0 Hz), 5.63, t, 1H, -CH_2N*H*-, *J* = 5.9 Hz), 5.68 (d, 1H, Gluc1-H, *J* = 7.9 Hz), 6.22 (d, 1H, 3'-NH-, *J* = 8.4 Hz), 7.36 (d, 1H, 3-H, *J* = 8.5 Hz), 7.76 (t, 1H, 2-H, *J* = 8.1 Hz), 8.00 (d, 1H, 1-H, *J* = 7.7 Hz), 13.23 (s, 1H, 11-OH), 13.96 (s, 1H, 6-OH).

N-γ-(Daunorubicin-3'-N-butyryl) O-β-D-glucuronyl carbamate sodium salt (**DAU-GB1**) from **46c** according to general procedure #6 in 98%, mp 188ºC (dec.), elem. anal.: calc. (found) for $C_{48}H_{43}N_2O_{19}Na\cdot 6\ H_2O$: C: 47.40 (47.02), H: 5.76 (5.18), N: 2.91 (3.05). MS (FAB$^+$) *m/z* = 856 ([M+1+H]$^+$), 855 ([M+H]$^+$). ^{1}H-NMR (100 MHz, $(CD_3)_2SO$) δ (ppm) = 1.11 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.30 (m, 3H, 2'$_{eq}$-H -$CH_2CH_2CH_2$-), 1.82 (td, 1H, 2'$_{ax}$-H, *J* = 12.1 Hz *J* = 3.6 Hz), 1.90-2.20 (m, 4H, -CH_2CH_2C(O)- 8$_{ax}$-H 8$_{eq}$-H), 2.25 (s, 3H, 9-C(O)Me), 2.95 (d, 1H, 10$_{eq}$-H, *J* = 9.8 Hz), 3.00-3.10 (m, 2H, -CH_2CH_2NH-), 3.16 (d, 1H, 10$_{ax}$-H, *J* = 9.8 Hz), 3.20-3.50 (m, 4H, 4'-H Gluc2,3,4-H), 3.97 (s, 3H, 4-OMe), 4.15 (q, 1H, 5'-H, *J* = 6.6 Hz), 4.94 (bs, 1H, 7-H), 5.02 and 5.07 (2s, 2H, 2 -OH), 5.12 (d, 1H, Gluc1-H, *J* = 8.1 Hz), 5.21 (bs, 1H, 1'-H), 5.53 (s, 1H, 9-OH), 7.25 (t, 1H, -CH_2CH_2N*H*-, *J* = 5.9 Hz), 6.63 (bs, 2H, 3-H 3'-NH-), 7.85-7.90 (m, 2H, 1-H 2-H), 13.27 (s, 1H, 11-OH), 14.04 (s, 1H, 6-OH).

Synthesis of *N*-γ-[daunorubicin-3'-*N*-(3,3-dimethyl)butyryl] *O*-β-D-glucuronyl carbamate sodium salt (DAU-GB2).

3,3-Dimethyl glutaric acid mono allyl ester (**27**) from **26** in 78% according to general procedure #1, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.15 (s, 6H, 2 -Me), 2.49 (s, 4H, 2 -CH_2-), 5.58 (dt, 2H, -OCH_2-, *J* = 6.0 Hz *J* = 1.2 Hz), 5.15-5.45 (m, 2H, =CH_2), 5.70-6.15 (m, 1H, -CH=), 11.51 (bs, 1H, -CO_2H).

N-γ-[(3,3-Dimethyl)butyric acid allyl ester] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**47a**) from **27** according to general procedure #2 as a white foam, mp 30-33ºC in 82% yield and 98% β-diastereoselectivity, ^{1}H-NMR (100 mHz, $CDCl_3$) δ (ppm) = 1.00 (s, 6H, -$C(Me)_2$-), 2.03 (s, 9H, 3 -OAc), 2.25 (s, 2H, -$C(Me)_2CH_2CO_2$-), 3.13 (d, 2H, -$C(Me)_2CH_2$NH-, *J* = 6.7 Hz), 3.74 (s, 3H, -CO_2Me), 4.16 (d, 1H, Gluc5-H, *J* = 9.8 Hz), 4.57 (d, 2H, -OCH_2CH=, *J* = 5.9 Hz), 5.10-5.95 (m, 6H, =CH_2 -CH= -NH- Gluc2,3,4-H), 5.72 (d, 1H, Gluc1-H, *J* = 8.1 Hz).

N-γ-[(3,3-Dimethyl)butyric acid] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**47b**) from **47a** according to general procedure #3.

N-γ-[Daunorubicin-3'-N-(3,3-dimethyl)butyryl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**47c**) from **47b** according to general procedure #5 in 24%, mp 148-150ºC, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 0.94 and 0.96 (2 s, 6H, 2 -CMe_2-), 1.30 (d, 3H, 5'-Me, *J* = 6.5 Hz), 1.75-1.95 (3H, 2'$_{ax}$-H, 2'$_{eq}$-H 4'-OH), 2.00,

2.02 and 2.04 (3 s, 9H, 3 -OAc), 2.00-2.20 (m, 3H, 8_{ax}-H, -$CMe_2CH_2C(O)$-), 2.02, 2.03 and 2.04 (3s, 9H, 3 -OAc), 2.32 (d, 1H, 8_{eq}-H, *J* = 14.8 Hz), 2.43 (s, 3H, 9-C(O)Me), 2.90 (d, 1H, 10_{ax}-H, *J* = 18.8 Hz), 3.02 (dd, 1H, -$CMe_2CH_aH_bNH$-, *J* = 14.1 Hz, *J* = 5.6 Hz), 3.13 (dd, 1H, -$CMe_2CH_aH_bNH$-, *J* = 14.1 Hz, *J* = 3.1 Hz), 3.25 (d, 1H, 10_{eq}-H, *J* = 18.8 Hz), 3.70 (s, 1H, 4'-H), 3.73 (s, 3H, C(O)OMe), 4.07 (s, 3H, 4-OMe), 4.14 (bs, 1H, 3'-H), 4.17 (d, 1H, Gluc5-H, *J* = 9.8 Hz), 4.22 (q, 1H, 5'-H, *J* = 5.6 Hz), 4.48 (s, 1H, 9-OH), 5.08 (t, 1H, Gluc2-H, *J* = 8.6 Hz), 5.19 (t, 1H, Gluc4-H, *J* = 9.6 Hz), 5.24 (s, 1H, 7-H), 5.31 (t, 1H, Gluc3-H, *J* = 9.3), 5.50 (d, 1H, 1'-H, *J* = 3.3 Hz), 5.70 (d, 1H, Gluc1-H, *J* = 8.0 Hz), 5.87 (t, 1H, -CH_2NH-, *J* = 6.6 Hz), 6.29 (d, 1H, 3'-NH-, *J* = 8.2 Hz), 7.38 (d, 1H, 3-H, *J* = 8.5 Hz), 7.77 (t, 1H, 2-H, *J* = 8.1 Hz), 8.01 (d, 1H, 1-H, *J* = 7.6 Hz), 13.25 (s, 1H, 11-OH), 13.97 (s, 1H, 6-OH).

N-γ-[Daunorubicin-3'-N-(3,3-dimethyl)butyryl] O-β-D-glucuronyl carbamate sodium salt (**DAU-GB2**) from **47c** according to general procedure #6 in 82%, mp 220ºC (dec.), elem. anal.: calc. (found) for $C_{40}H_{47}N_2O_{19}Na{\cdot}6\,H_2O$: C: 48.49 (48.03), H: 6.00 (5.74), N: 2.83 (2.92). MS (FAB^+) *m/z* = 906 ($[M+1+Na]^+$), 905 ($[M+Na]^+$), 884 ($[M+1+H]^+$), 883 ($[M+H]^+$). ^{1}H-NMR (100 MHz, $(CD_3)_2SO$) δ (ppm) = 0.86 (s, 6H, -CMe_2-), 1.13 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.41 (dd, 1H, $2'_{eq}$-H, *J* = 12.1 Hz *J* = 3.6 Hz), 1.82 (td, 1H, $2'_{ax}$-H, *J* = 12.1 Hz *J* = 3.6 Hz), 1.98 (s, 2H, -$C(Me)_2CH_2C(O)$-), 2.13 (dd, 1H, 8_{ax}-H, *J* = 14.3 Hz *J* = 5.6 Hz), 2.20 (bd, 1H, 8_{eq}-H, *J* = 13.9), 2.26 (s, 3H, 9-C(O)Me), 2.87 (d, 2H, -$C(Me)_2CH_2NH$-, *J* = 5.8), 2.90-3.55 (m, 6H, 10_{eq}-H 10_{ax}-H 4'-H Gluc2,3,4-H), 3.90-4.00 (m, 3H, 3'-H 2 -OH), 3.98 (s, 3H, 4-OMe), 4.15 (q, 1H, 5'-H, *J* = 6.6 Hz), 4.92 (s, 1H, -OH), 5.02 (s, 1H, -OH), 4.97 (bs, 1H, 7-H), 5.18 (d, 1H, Gluc1-H, *J* = 7.9 Hz), 5.24 (bs, 1H, 1'-H), 5.46 (s, 1H, 9-OH), 7.29 (t, 1H, -CH_2NH-, *J* = 5.9 Hz), 7.55-7.65 (m, 2H, 3-H 3'-NH-), 7.85-7.90 (m, 2H, 1-H 2-H), 13.25 (s, 1H, 11-OH), 14.00 (s, 1H, 6-OH).

Synthesis of *N*-γ-[daunorubicin-3'-*N*-(4,4-dimethyl)butyryl] *O*-β-D-glucuronyl carbamate sodium salt (DAU-GB3).

4,4-Dimethyl glutaric acid mono allyl ester (**29**), together with a small amount of the 2,2-dimethyl isomer **30** [15] obtained almost quantitatively from **28** according to general procedure #1. **29**: ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.23 (s, 6H, 2 -Me), 1.60-2.10 (m, 2H, -$C(Me)_2CH_2$-), 2.20-2.50 (m, 2H, -CH_2CO_2-), 4.57 (dt, 2H, -OCH_2-, *J* = 5.3 Hz *J* = 1.2 Hz), 5.15-5.40 (m, 2H, =CH_2), 5.75-6.15 (m, 1H, -CH=), 10.55 (bs, 1H, -CO_2H).

N-γ-[(4,4-{And 2,2-}dimethyl)butyric acid allyl ester] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**48a** {and **49a**}) from **29** {and **30**} according to general procedure #2 in 81% yield and 100% β-diastereoselectivity as a colorless oil, **48a**: ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.25 (s, 3H, -CMe_aMe_b-), 1.29 (s, 3H, -CMe_aMe_b-), 2.00 (s, 9H, 3 -OAc), 2.25-2.45 (m, 2H, -$C(Me)_2CH_2CO_2$-), 3.71 (s, 3H, -CO_2Me), 4.14 (d, 1H, Gluc5-H, *J* = 9,1 Hz), 4.56 (d, 2H, -OCH_2CH=, *J* = 5.8 Hz), 5.05-5.45 (m, 6H, =CH_2 -NH- Gluc2,3,4-H), 5.65-6.10 (m, 1H, -CH=), 5.66 (d, 1H, Gluc1-H, *J* = 7.8 Hz).

N-γ-[(4,4-{And 2,2-}dimethyl)butyric acid] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**48b** {and **49b**}) from **48a** {and **49a**} according to general procedure #3.

N-γ-[Daunorubicin-3'-N-(4,4-{and 2,2-}dimethyl)butyryl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**48c** {and **49c**}) from **48b** {and **49b**} according to general procedure #5 in 58%. Both isomers were separated and obtained in a ratio of 7.3/1. For both isomers mp 148-150ºC. **48c**: ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.24 and 1.28 (2 s, 6H, 2 -CMe_2-), 1.30 (d, 3H, 5'-Me, *J* = 6.7 Hz), 1.75-2.20 (m, 8H, $2'_{ax}$-H $2'_{eq}$-H 4'-OH 8_{ax}-H -$CH_2CH_2C(O)$- -$CH_2CH_2C(O)$-), 2.03 (s, 9H, 3 -OAc), 2.32 (d, 1H, 8_{eq}-H, *J* = 14.8 Hz), 2.42 (s, 3H, 9-C(O)Me), 2.90 (d, 1H, 10_{ax}-H, *J* = 18.8 Hz), 3.22 (d, 1H, 10_{eq}-H, *J* = 18.8 Hz), 3.67 (s, 1H, 4'-H), 3.73 (s, 3H, C(O)OMe), 4.07 (s, 3H, 4-OMe), 4.14 (bs, 1H, 3'-H), 4.16 (d, 1H, Gluc5-H, *J* = 9.8 Hz), 4.22 (q, 1H, 5'-H, *J* = 6.6 Hz), 4.50 (s, 1H, 9-OH), 5.07 (t, 1H, Gluc2-H, *J* = 8.5 Hz), 5.19 (t, 1H, Gluc4-H, *J* = 9.6 Hz), 5.24 (s, 1H, 7-H), 5.30 (t, 1H, Gluc3-H, *J* = 9.3), 5.36 (s, 1H, -CMe_2NH-), 5.49 (bs, 1H, 1'-H), 5.65 (d, 1H, Gluc1-H, *J* = 7.8 Hz), 6.08 (d, 1H, 3'-NH-, *J* = 8.2 Hz), 7.38 (d, 1H, 3-H, *J* = 8.5 Hz), 7.77 (t, 1H, 2-H, *J* = 8.1 Hz), 8.01 (d, 1H, 1-H, *J* = 7.6 Hz), 13.26 (s, 1H, 11-OH), 13.97 (s, 1H, 6-OH).
49c: ^{1}H-NMR spectrum almost similar to that of **48c** except for the absence of the -$CH_2CH_2C(O)$- signal, the presence of the signal at 3.00-3.10 (m, 2H, -CH_2CH_2NH-) and the -CH_2NH- overlapping with other signals.

N-γ-[daunorubicin-3'-N-(4,4-dimethyl)butyryl] O-β-D-glucuronyl carbamate sodium salt (**DAU-GB3**) from **48c** according to general procedure #6 in 96%, mp 200ºC (dec.), elem. anal.: calc. (found) for $C_{40}H_{47}N_2O_{19}\cdot 6\ H_2O$: C: 48.49 (48.22), H: 6.00 (5.65), N: 2.83 (2.88). MS (FAB$^+$) *m/z* = 906 ([M+1+Na]$^+$), 905 ([M+Na]$^+$), 884 ([M+1+H]$^+$), 883 ([M+H]$^+$). ^{1}H-NMR (100 MHz, $(CD_3)_2SO$) δ (ppm) = 1.10 and 1.18 (2s, 6H, -CMe_2-), 1.13 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.43 (bd, 1H, 2'$_{eq}$-H, *J* = 8.4 Hz), 1.60-2.20 (m, 7H, -$C(Me)_2CH_2CH_2$- 2'$_{ax}$-H 8$_{ax}$-H 8$_{eq}$-H -$CH_2CH_2C(O)$-), 2.25 (s, 3H, 9-C(O)Me), 2.90-3.25 (m, 6H,- 10$_{eq}$-H 10$_{ax}$-H 4'-H Gluc2,3,4-H), 3.96 (s, 3H, 4-OMe), 4.00-4.05 (m, 2H, 3'-H Gluc5-H), 4.15 (q, 1H, 5'-H, *J* = 6.6 Hz), 4.91 (s, 1H, -OH), 5.00-5.05 (m, 3H, 7-H, 2 -OH), 5.03 (d, 1H, -OH, *J* = 5.4 Hz), 5.09 (d, 1H, Gluc1-H, *J* = 8.4 Hz), 5.20 (bs, 1H, 1'-H), 5.55 (s, 1H, 9-OH), 7.06 (s, 1H, -CMe_2N*H*-), 7.61 (bs, 1H, 3-H), 7.76 (d, 1H, 3'-NH-, *J* = 8.0 Hz),7.88 (bs, 2H, 1-H 2-H), 13.18 (s, 1H, 11-OH), 13.99 (s, 1H, 6-OH).

N-γ-[daunorubicin-3'-N-(2,2-dimethyl)butyryl] O-β-D-glucuronyl carbamate sodium salt (**DAU-GB11**) from **49c** according to general procedure #6 in 37%, mp 193ºC (dec.), elem. anal.: calc. (found) for $C_{40}H_{47}N_2O_{19}\cdot 6\ H_2O$: C: 48.49 (48.15), H: 6.00 (5.91), N: 2.83 (2.86). (The low yield compared to the deprotection leading to **DAU-GB3** was caused by mechanical loss during this small scale deprotection procedure). MS (FAB$^+$) *m/z* = 906 ([M+1+Na]$^+$), 905 ([M+Na]$^+$), 884 ([M+1+H]$^+$), 883 ([M+H]$^+$). ^{1}H-NMR (100 MHz, $(CD_3)_2SO$) δ (ppm) = 1.02 and 1.06 (2s, 6H, -CMe_2-), 1.13 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.44 (dd, 1H, 2'$_{eq}$-H, *J* = 12.2 Hz *J* = 4.0 Hz), 1.50-1.70 (m, 2H, -$C(Me)_2CH_2CH_2$-), 1.90 (td, 1H, 2'$_{ax}$-H, *J* = 12.6 Hz *J* = 3.9 Hz), 2.11 (dd, 1H, 8$_{ax}$-H, *J* = 14.2 Hz *J* = 5.5 Hz), 2.19 (bd, 1H, 8$_{eq}$-H, *J* = 14.0), 2.26 (s, 3H, 9-C(O)Me), 2.85-3.15 (m, 8H, -CH_2CH_2NH- 10$_{eq}$-H 10$_{ax}$-H Gluc2,3,4-H), 3.43 (s, 1H, 4'-H), 3.98 (s, 3H, 4-OMe), 4.00-4.05 (m, 2H, 3'-H Gluc5-H), 4.16 (q, 1H, 5'-H, *J* = 6.5 Hz), 4.90-5.00 (m, 3H, 7-H, 2 -OH), 5.03 (d, 1H, -OH, *J* = 5.4 Hz), 5.13 (d, 1H, Gluc1-H, *J* = 8.2 Hz), 5.24 (bs, 1H, 1'-H), 5.55 (s, 1H, 9-OH), 6.83 (d, 1H, 3'-NH-, *J* = 6.8 Hz), 7.13 (t, 1H, -CH_2N*H*-, *J* = 5.5 Hz), 7.64 (bs, 1H, 3-H), 7.85-7.95 (m, 2H, 1-H 2-H), 13.27 (s, 1H, 11-OH), 14.03 (s, 1H, 6-OH).

Attempted synthesis of *N*-2-[2'-(daunorubicin-3'-*N*-carbonyl)]biphenyl *O*-β-D-glucuronyl carbamate sodium salt (DAU-GB4).

Diphenic acid mono allyl ester (**32**) obtained quantitatively from **31** according to general procedure #1, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 4.42 (d, 2H, -OCH_2-, *J* = 5.6 Hz), 4.90-5.15 (m, 2H, =CH_2), 5.40-5.80 (m, 1H, -CH=), 7.00-7.55 (m, 4H, Ar3,3',4,4',5,5'-H), 7.90-8.00 (m, 2H, Ar6,6'-H), 10.83 (s, 1H, -CO_2H).

N-2-[Biphenyl-2'-carboxylic acid allyl ester] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**50a**) from **32** according to general procedure #2 as white needles from *i*-Pr_2O/EtOAc, mp 63-67ºC in 89% yield and 100% β-diastereoselectivity, the ^{1}H-NMR spectrum of **50a** taken on a 400 mHz recorder in $CDCl_3$ almost all signals were double, indicating the existence of two rotameric forms of the compound in a ratio of 4/5. This was likely due to restricted rotation around the Ar-Ar bond. These double signals coalesce upon heating. δ (ppm) = 1.95-2.05 (m, 9H, 3 -OAc), 3.72 and 3.73 (s, 3H, -CO_2Me), 4.17 (d, 1H, Gluc5-H, *J* = 9.6 Hz), 4.49 and 4.53 (2d, 2H, -OCH_2CH=, *J* = 5.8 resp. 5.2 Hz), 5.00-5.35 (m, 5H, =CH_2 Gluc2,3,4-H), 5.55-5.75 (m, 1H, -CH=), 5.73 and 5.74 (2d, 1H, Gluc1-H, *J* = 8.0 resp. 8.1 Hz), 6.45 and 6.51 (s, 1H, -NH-), 7.05-7.65 (m, 6H, Ar3,3',4,4',5,5'-H), 7.85-8.05 (m, 2H, Ar6,6'-H).

N-2-[Biphenyl-2'-carboxylic acid] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**50b**) from **50a** according to general procedure #3 in 93%, mp 90-92ºC, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.70-2.00 (m, 9H, 3 -OAc), 3.64 and 3.67 (s, 3H, -CO_2Me), 4.06 and 4.13 (2d, 1H, Gluc5-H, *J* = 9.0 resp. 9.2 Hz), 4.85-5.35 (m, 3H, Gluc2,3,4-H), 5.63 and 5.66 (2d, 1H, Gluc1-H, *J* = 7.6 resp. 7.9 Hz), 6.62 and 6.70 (2s, 1H, -NH-), 7.10-8.00 (m, 8H, Ar3,3',4,4',5,5',6,6'-H).

The coupling reaction of the above carboxylic acid **50b** with daunorubicin using a number of different coupling procedures all resulted in the formation of compound **52** (see scheme 3.9), This reaction is further outlined in section 5.2, The desired product could not be obtained.

Synthesis of *N*-methyl, *N*-2-[2'-(daunorubicin-3'-*N*-carbonyl)]biphenyl *O*-β-D-glucuronyl carbamate sodium salt (DAU-Me-GB4).

N-Methyl, N-2-[biphenyl-2'-carboxylic acid allyl ester] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**54a**). 100 mg of **50a** was dissolved in 4 mL of DMF and 57 mg (1.5 equiv.) of Ag_2O and 101 μL (10 equiv.) of MeI were added. The suspension was stirred for 4 h at ambient temperature and after that filtered and diluted with 100 mL of EtOAc. The solution was washed 50 mL portions of aqueous 5% $KHSO_4$ with saturated aqueous $NaHCO_3$ (2x) and with brine and dried over Na_2SO_4. The product was purified by means of column chromatography (SiO_2, Et_2O/*n*-Hex 3/1) to obtain 42 mg (56%) of **54a** as an oil (after column chromatography, 27 mg starting material was recovered). Extremely broad signals were obtained in the ^{1}H-NMR spectrum [29], (100 MHz, $CDCl_3$) δ (ppm) = 1.80-2.05 (m, 9H, 3 -OAc), 2.61 and 2.76 (2bs, 3H, -NMe-), 3.67 (bs, 3H, -CO_2Me), 4.09 (bd, 1H, Gluc5-H, *J* = 9.5 Hz), 4.46 (bd, 2H, -OC*H*$_2$CH=, *J* = 5.4 Hz), 4.80-5.35 (m, 5H, =CH_2 Gluc2,3,4-H), 5.35-5.80 (m, 2H, Gluc1-H -CH=), 6.95-7.65 (m, 6H, Ar3,3',4,4',5,5'-H), 7.70-8.10 (m, 2H, Ar6,6'-H).

N-Methyl, N-2-[biphenyl-2'-carboxylic acid] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**54b**) from **54a** according to general procedure #3.

N-methyl, N-2-[2'-(daunorubicin-3'-N-carbonyl)]biphenyl O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**54c**) from **54b** according to general procedure #5 in 73%, mp 150-165ºC. ^{1}H-NMR [29] (400 MHz, $CDCl_3$) δ (ppm) = 1.10-1.30 (m, 3H, 5'-Me), 1.55-2.35 (m, 14H, 2'$_{ax}$-H 2'$_{eq}$-H 4-OH 8$_{ax}$-H 8$_{eq}$-H 3 -OAc), 2.42 and 2.43 (2s, 3H, 9-C(O)Me), 2.99 (d, 1H, 10$_{ax}$-H, *J* = 18.8 Hz), 3.04 and 3.35 (2s, 3H, ArN*Me*-), 3.23 (d, 1H, 10$_{eq}$-H, *J* = 18.8 Hz), 3.40-4.15 (m, 4H, 4'-H Gluc5-H 5'-H -CO_2Me 4-OMe), 4.59 (s, 1H, 9-OH), 5.05-5.45 (m, 6H, Gluc1,2,3,4-H 7-H 1'-H 3'-NH-), 7.05-7.70 (m, 9H, 3-H Ar3,3',4,4',5,5',6,6'-H), 7.78 (t, 1H, 2-H, *J* = 8.0 Hz), 8.05 (d, 1H, 1-H, *J* = 7.6 Hz), 13.29 and 13.30 (2s, 1H, 11-OH), 13.92, 13.95 and 13.96 (3s, 1H, 6-OH).

N-methyl, N-2-[2'-(daunorubicin-3'-N-carbonyl)]biphenyl O-β-D-glucuronyl carbamate (**Dau-Me-GB4**) from **54c** according to general procedure #6 in 49%, mp 175-177ºC, elem. anal.: calc. (found) for $C_{48}H_{47}N_2O_{19}\cdot 5\,H_2O$: C: 53.93 (53.71), H: 5.37 (5.14), N: 2.62 (2.69). ^{1}H-NMR [29] (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.10-1.20 (m, 3H, 5'-Me), 1.47-2.20 (m, 4H, 2'$_{eq}$-H 2'$_{ax}$-H 8$_{ax}$-H 8$_{eq}$-H), 2.28 (bs, 3H, -C(O)Me), 3.10-3.15 (m, 2H, 10$_{eq}$-H 10$_{ax}$-H), 3.20-4.80 (m, 14H, 4'-H Gluc2,3,4,5-H 3'-H 4-OMe 5'-H 4'-OH ArN*Me*-), 4.90-5.30 (m, 3H, 7-H 1'-H Gluc1-H), 5.54 (s, 1H, 9-OH), 6.80-7.90 (m, 12H, 1,2,3-H 3'-NH- Ar3,3',4,4',5,5',6,6'-H) 13.20 (bs, 1H, 11-OH), 13.96 (bs, 1H, 6-OH).

Synthesis of *N*-2-(daunorubicin-3'-*N*-carbonylmethylphenyl) *O*-β-D-glucuronyl carbamate sodium salt (DAU-GB5).

Homophthalic acid ω-allyl ester (**34**) from **33** according to general procedure #1 after crystallization from *n*-Hex in 86%, mp 77-79ºC, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 4.01 (s, 1H, ArC*H*$_2$-), 4.57 (d, 2H, -OC*H*$_2$-, *J* = 6.9 Hz), 5.05-5.40 (m, 2H, =CH_2), 5.60-6.15 (m, 1H, -CH=), 7.10-7.65 (m, 3H, Ar3,4,5-H), 8.12 (d, 1H, Ar1-H, *J* = 8.1 Hz), 10 66 (s, 1H, -CO_2H).

N-2-Phenylacetic acid allyl ester O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**55a**) from **34** according to general procedure #2 in 86% yield and 100% β-diastereoselectivity as white needles from *i*-Pr_2O/EtOAc, mp 82ºC, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.98 (s, 9H, 3 -OAc), 3.51 (d, 1H, ArC*H*$_a$H$_b$-, *J* = 14 Hz), 3.66 (d, 1H, ArH$_a$*H*$_b$-, *J* = 14 Hz), 3.67 (s, 3H, -CO_2Me), 4.15 (d, 1H, Gluc5-H, *J* = 9.2 Hz), 4.51 (dt, 2H, -OC*H*$_2$CH=, *J* = 1.2 Hz *J* = 5.7 Hz), 5.00-5.45 (m, 5H, =CH_2 Gluc2,3,4-H), 5.60-6.10 (m, 1H, =CH_2), 5.76 (d, 1H, Gluc1-H, *J* = 7.3 Hz), 7.00-7.40 (m, 3H, Ar3,4,5-H), 7.64 (d, 1H, Ar6-H, *J* = 7.5 Hz), 8.04 (s, 1H, -NH-).

N-2-Phenylacetic acid O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**55c**) from **55a** according to general procedure #3 in an almost quantitative yield. Compound **55c** proved to be unstable and was coupled to daunorubicin immediately.

N-2-(Daunorubicin-3'-N-carbonylmethylphenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**55d**) from **55c** according to general procedure #5 in 32%, mp 162-165°C, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.33 (d, 3H, 5'-Me, *J* = 6.5 Hz), 1.70 (m, 4H, 2'$_{ax}$-H 2'$_{eq}$-H 8$_{ax}$-H 4'-OH), 2.05, 2.08 and 2.09 (3 s, 9H, 3 -OAc), 2.36 (d, 1H, 8$_{eq}$-H, *J* = 14.9 Hz), 2.44 (s, 3H, 9-C(O)Me), 2.96 (d, 1H, 10$_{ax}$-H, *J* = 18.9 Hz), 3.25 (d, 1H, 10$_{eq}$-H, *J* = 18.8 Hz), 3.69 (s, 1H, 4'-H), 3.73 (d, 1H, ArC*H$_a$*H$_b$-, *J* = 12.4 Hz), 3.74 (s, 3H, C(O)OMe), 3.77 (d, 1H, ArH$_a$*H$_b$*-, *J* = 12.4 Hz),3.88 (m, 1H, 3'-H), 4.07 (s, 3H, 4-OMe), 4.11 (d, 1H, Gluc5-H, *J* = 9.6 Hz), 4.29 (q, 1H, 5'-H, *J* = 6.6 Hz), 4.55 (s, 1H, 9-OH), 5.10-5.35 (m, 5H, Gluc2,3,4-H 7-H 3'-NH-), 5.55 (d, 1H, 1'-H, *J* = 3.8 Hz), 5.60 (d, 1H, Gluc1-H, *J* = 9.7 Hz), 7.21 (s, 1H, ArNH-), 7.11 (t, 1H, Ar5-H, *J* = 7.5 Hz), 7.22 (t, 1H, Ar4-H, *J* = 7.2 Hz) 7.38 (d, 1H, 3-H, *J* = 8.5 Hz), 7.78 (t, 1H, 2-H, *J* = 8.1 Hz), 8.04 (d, 1H, 1-H, *J* = 7.7 Hz), 8.16 (d, 1H, Ar3-H, *J* = 8.2 Hz), 8.86 (d, 1H, Ar6-H, *J* = 7.8 Hz), 13.29 (s, 1H, 11-OH), 13.98 (s, 1H, 6-OH).

N-2-(Daunorubicin-3'-N-carbonylmethylphenyl) O-β-D-glucuronyl carbamate sodium salt (**DAU-GB5**) from **55d** according to general procedure #6 in 62%, mp 180°C (dec.), elem. anal.: calc. (found) for $C_{42}H_{43}N_2O_{19}\cdot 6\,H_2O$: C: 49.90 (50.34), H: 5.48 (4.88), N: 2.77 (2.82). MS (FAB$^+$) *m/z* = 926 ([M+1+Na]$^+$), 925 ([M+Na]$^+$), 904 ([M+1+H]$^+$), 903 ([M+H]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.13 (d, 3H, 5'-Me, *J* = 6.6 Hz), 1.47 (dd, 1H, 2'$_{eq}$-H, *J* = 12.1 Hz *J* = 3.4 Hz), 1.82 (dt, 1H, 2'$_{ax}$-H, *J* = 11.5 Hz *J* = 3.4 Hz), 2.07 (dd, 1H, 8$_{ax}$-H, *J* = 14.1 Hz *J* = 6.1 Hz), 2.19 (dd, 1H, 8$_{eq}$-H, *J* = 14.1 *J* = 3.1 Hz), 2.27 (s, 3H, -C(O)Me), 3.11 (d, 1H, 10$_{eq}$-H, *J* = 18.5 Hz), 3.13 (d, 1H, 10$_{ax}$-H, *J* = 18.5 Hz), 3.20-3.75 (m, 7H, 3',4'-H Gluc2,3,4-H ArC*H$_2$*-), 3.95 (s, 3H, 4-OMe), 4.19 (q, 1H, 5'-H, *J* = 6.6 Hz), 4.67 (d, 1H, 4'-OH, *J* = 5.1 Hz), 4.75-5.35 (m, 4H, Gluc1,5-H 7-H 1'-H), 5.53 (s, 1H, 9-OH), 5.76 (d, 1H, 3'-NH-, *J* = 7.9 Hz), 6.78 (t, 1H, Ar5-H, *J* = 7.3 Hz), 6.98 (t, 1H, Ar4-H, *J* = 6.7 Hz), 7.25 (s, 1H, ArN*H*-), 7.30 (d, 1H, Ar3-H, *J* = 8.4 Hz), 7.67 (d, 1H, 3-H, *J* = 8.1 Hz), 7.82 (d, 1H, Ar6-H, *J* = 8.3 Hz), 7.94 (m, 2H, 1,2-H), 13.23 (s, 1H, 11-OH), 13.97 (s, 1H, 6-OH).

Synthesis of *N*-2-(daunorubicin-3'-*N*-carbonylbenzyl) *O*-β-D-glucuronyl carbamate sodium salt (DAU-GB8).

Homophthalic acid ω-allyl- α-tert.-butyl diester (**35**). 538 mg of **34** (obtained as described above under **DAU-GB5**) was dissolved in 25 mL of CH_2Cl_2 and cooled to -10°C. *Ca.* 2 mL of condensed isobutene and 3 drops of concentrated H_2SO_4 were added to the reaction mixture which was stirred overnight in an autoclave at room temperature. After reaction, the H_2SO_4 was neutralized using excess of solid $NaHCO_3$, the mixture was filtered and the solvent was removed by evaporation. The residue was dissolved in *n*-Hex, the unreacted starting material precipitated from this solution and was removed and the hexane was removed evaporated to furnish 478 mg, 77% of **35** (corrected for the recovered **34**) as a colorless oil, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.46 (s, 9H, -*t*-Bu), 3.95 (s, 2H, ArC*H$_2$*-), 4.49 (d, 2H, -OCH$_2$-, *J* = 5.5 Hz), 5.00-5.30 (m, 2H, =CH$_2$), 5.60-6.00 (m, 1H, -CH=), 7.05-7.40 (m, 3H, Ar3,4,5-H), 7.83 (dd, 1H, Ar6-H, *J* = 7.2 Hz *J* = 2.1 Hz).

Homophthalic acid α-tert.-butyl ester (**36**) from **35** according to general procedure #3 in quantitative yield.

N-[Benzyl-2-carboxylic acid tert.-butyl ester] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**56b**) from **36** according to general procedure #2 in 68% yield and >95% β-diastereoselectivity as white needles from Et_2O, mp 112-119°C, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.53 (s, 9H, *tert.*-Bu), 1.91, 1.94 and 1.95 (3s, 9H, 3 -OAc), 3.63 (s, 3H, -CO$_2$Me), 4.08 (d, 1H, Gluc5-H, *J* = 9.4 Hz), 4.45 (d, 2H, ArC*H$_2$*-, *J* = 6.6 Hz), 4.90-5.45 (m, 3H, Gluc2,3,4-H), 5.66 (d, 1H, Gluc1-H, *J* = 7.7 Hz), 6.07 (t, 1H, -NH-, *J* = 6.6 Hz), 7.10-7.50 (m, 3H, Ar3,4,5-H), 7.85 (d, 1H, Ar6-H, *J* = 6.8 Hz).

N-[Benzyl-2-carboxylic acid] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**56c**) from **56b** according to general procedure #4 in quantitative yield (according to TLC). **56c** was coupled to daunorubicin immediately to prevent deterioration.

N-2-(Daunorubicin-3'-N-carbonylbenzyl) O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**56d**) from **56c** according to general procedure #5 in 62% (calculated from **56b**), mp 164-168, ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.35 (d, 3H, 5'-Me, *J* = 6.5 Hz), 1.75-2.05 (m, 3H, 2'$_{ax}$-H 2'$_{eq}$-H 4-OH), 1.96, 2.01 and 2.02 (3 s, 9H, 3 -OAc), 2.14 (dd, 1H, 8$_{ax}$-H, *J* = 14.8 Hz *J* = 4.0 Hz), 2.38 (d, 1H, 8$_{eq}$-H, *J* = 14.5 Hz), 2.46 (s, 3H, 9-C(O)Me), 2.94 (d, 1H, 10$_{ax}$-H, *J* = 18.8 Hz), 3.25 (d, 1H, 10$_{eq}$-H, *J* = 18.8 Hz), 3.63 (s, 3H, -CO$_2$Me), 3.83 (s,

1H, 4'-H), 4.07 (s, 3H, 4-OMe), 4.11 (d, 1H,Gluc5-H, *J* = 9.8 Hz), 4.25-4.45 (m, 4H, 5'-H ArCH_2- 3'-H), 4.56 (s, 1H, 9-OH), 5.02 (t, 1H, Gluc2-H, *J* = 8.4 Hz), 5.16 (t, 1H, Gluc4-H, *J* = 9.6 Hz), 5.27 (t, 1H, Gluc3-H, *J* = 9.3 Hz), 5.30 (s, 1H, 7-H), 5.57 (s, 1H, 1'-H), 5.64 (d, 1H, Gluc1-H, *J* = 7.7 Hz), 6.40-6.45 (m, 2H, 3'-NH-ArCH$_2$N*H*-), 7.25-7.40 (m, 5H, 3-H Ar3,4,5,6-H), 7.77 (t, 1H, 2-H, *J* = 8.1 Hz), 8.00 (d, 1H, 1-H, *J* = 7.7 Hz), 13.27 (s, 1H, 11-OH), 14.00 (s, 1H, 6-OH).

N-2-(Daunorubicin-3'-N-carbonylbenzyl) O-β-D-glucuronyl carbamate sodium salt (**DAU-GB8**) from **56d** in 77%, mp 190ºC (dec.), elem. anal.: calc. (found) for $C_{42}H_{43}N_2O_{19}$·6 H_2O: C: 49.90 (50.27), H: 5.48 (4.92), N: 2.77 (2.80). MS (FAB$^+$) *m/z* = 926 ([M+1+Na]$^+$), 925 ([M+Na]$^+$), 904 ([M+1+H]$^+$), 903 ([M+H]$^+$). ^{1}H-NMR (100 MHz, $(CD_3)_2SO$) δ (ppm) = 1.15 (d, 3H, 5'-Me, *J* = 6.5 Hz), 1.54 (dd, 1H, 2'$_{eq}$-H, *J* = 12.1 Hz *J* = 3.9 Hz), 1.99 (dt, 1H, 2'$_{ax}$-H, *J* = 12.9 Hz *J* = 3.4 Hz), 2.11 (dd, 1H, 8$_{ax}$-H, *J* = 12.9 Hz *J* = 3.4 Hz), 2.22 (dd, 1H, 8$_{eq}$-H, *J* = 13.2 *J* = 2.6 Hz), 2.27 (s, 3H, -C(O)Me), 2.90-3.20 (m, 6H, 10$_{eq}$-H 10$_{ax}$-H 4'-H Gluc2,3,4-H), 3.61 (bs, 1H, 3'-H), 3.97 (s, 3H, 4-OMe), 4.15-4.35 (m, 4H, 5'-H Gluc5-H ArCH_2-), 4.95-5.00 (m, 2H, 7-H ArCH$_2$N*H*-), 5.08 (d, 1H, 4'-OH, *J* = 5.2 Hz), 5.13 (d, 1H, Gluc1-H, *J* = 8.1 Hz), 5.27 (bs, 1H, 1'-H), 5.59 (s, 1H, 9-OH), 7.20-7.40 (m, 3H, Ar3,5-H 3'-NH-), 7.55-7.65 (m, 2H, 3-H Ar4-H), 7.85-7.90 (m, 2H, 1,2-H), 7.97 (d, 1H, Ar6-H, *J* = 7.8 Hz), 14.03 (s, 1H, 6-OH).

Synthesis of *N*-2-(daunorubicin-3'-*N*-carbonyl-(α-methyl)benzyl) *O*-β-D-glucuronyl carbamate sodium salt (DAU-GB9).

α-Methylhomophthalic acid ω-allyl α-tert.-butyl diester (**43**) from **35** according to general procedure #7 in 41%, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.40-1.60 (m, 12H, -*t*-Bu -Me), 4.50 (dt, 2H, -OCH$_2$-, *J* = 5.0 Hz *J* = 1.3 Hz), 4.61 (q, 1H, ArC*H*Me-, *J* = 7.1 Hz), 4.95-5.25 (m, 2H, =CH$_2$), 5.55-5.95 (m, 1H, -CH=), 7.10-7.50 (m, 3H, Ar3,4,5-H), 7.73 (dd, 1H, Ar6-H, *J* = 8.2 Hz *J* = 1.6 Hz).

α-Methylhomophthalic acid α-tert.-butyl ester (**45**) from **43** according to general procedure #3 in quantitative yield.

N-(α-Methylbenzyl-2-carboxylic acid -tert.-butyl ester) O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**60b**) from **45** according to general procedure #2 in 62% yield and 100% β-diastereoselectivity as a white foam, mp 69-74ºC. Because the starting material **45** was used as a racemic mixture, **60b** was formed as a mixture of diastereoisomers, these could not be separated. In the ^{1}H-NMR of this diastereoisomeric mixture, some signals were doubled and some were broadened. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.15-1.30 (m, 3 H. -CH*Me*-), 1.36 (s, 9H, *t*-Bu), 1.66, 1.76, 1.78 and 1.81 (s, 9H, 3 -OAc), 3.41 and 3.49 (s, 3H, -CO$_2$Me), 3.86 and 3.91 (d, 1H, Gluc5-H, *J* = 9.4 and 9.1 Hz resp.), 4.75-5.30 (m, 4H, ArC*H*Me- Gluc2,3,4-H), 5.44 and 5.46 (d, 1H, Gluc1-H, *J* = 7.4 and 7.6 Hz resp.), 6.00 (t, 1H, -NH-, *J* = 8.6 Hz), 6.90-7.30 (m, 3H, Ar3,4,5-H), 7.53 (d, 1H, Ar6-H, *J* = 6.4 Hz).

N-(α-Methylbenzyl-2-carboxylic acid) O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**60c**) from **60b** according to general procedure #4 in quantitative yield (according to TLC). **60c** was coupled to daunorubicin immediately to prevent deterioration.

N-2-(Daunorubicin-3'-N-carbonyl-α-methylbenzyl) O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**60d**) from **60c** according to general procedure #5 in 86% (calculated from **60b**). Because of chirality of the spacer -C*HMe- of starting material **60d**, a diastereoisomeric mixture was formed in a ratio of 1.8/1, determined by dividing the integrals of the clear doublets of the respective 3'-NH- signals. For both diastereoisomers, mp 165-170ºC. The isomers were separated by 3 successive runs of circular chromatography using CH_2Cl_2/EtOH 40/1. Major diastereoisomer: ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.32 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.46 (d, 3H, ArCH*Me*NH-, *J* = 6.9), 1.85-1.95 (m, 2H, 2'$_{ax}$-H 2'$_{eq}$-H), 1.90, 2.01 and 2.04 (3 s, 9H, 3 -OAc), 2.13 (dd, 1H, 8$_{ax}$-H, *J* = 14.8 Hz *J* = 4.0 Hz), 2.38 (d, 1H, 8$_{eq}$-H, *J* = 14.8 Hz), 2.44 (s, 3H, 9-C(O)Me), 2.93 (d, 1H, 10$_{ax}$-H, *J* = 18.8 Hz), 3.24 (d, 1H, 10$_{eq}$-H, *J* = 18.6 Hz), 3.33 (d, 1H, 4'-OH, *J* = 6.0 Hz), 3.73 (s, 3H, -CO$_2$Me), 3.92 (bd, 1H, 4'-H, *J* = 3.2 Hz), 4.09 (s, 3H, 4-OMe), 4.15 (d, 1H,Gluc5-H, *J* = 9.0 Hz), 4.27 (q, 1H, 5'-H, *J* = 6.6 Hz), 4.32 (bs, 1H, 3'-H), 4.57 (s, 1H, 9-OH), 5.00-5.25 (m, 4H, Gluc2,3,4-H ArC*H*MeNH-), 5.30 (s, 1H, 7-H), 5.54 (s, 1H, Gluc1-H, *J* = 7.9 Hz), 5.57 (d, 1H, 1'-H, *J* = 3.5 Hz), 5.91 (d, 1H, ArCHMeN*H*-, *J* = 6.7 Hz), 6.48 (d, 1H, 3'-NH-, *J* = 8.4 Hz), 7.20-7.40 (m, 5H, 3-H

Ar3,4,5,6-H), 7.78 (t, 1H, 2-H, *J* = 8.1 Hz), 8.03 (d, 1H, 1-H, *J* = 7.7 Hz), 13.28 (s, 1H, 11-OH), 14.00 (s, 1H, 6-OH).
Minor diastereoisomer: ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.37 (d, 3H, 5'-Me, *J* = 6.5 Hz), 1.55 (d, 3H, ArCH*Me*NH-, *J* = 7.1), 1.80-2.00 (m, 2H, 2'$_{ax}$-H 2'$_{eq}$-H), 2.03, 2.04 and 2.05 (3 s, 9H, 3 -OAc), 2.15 (dd, 1H, 8$_{ax}$-H, *J* = 15.0 Hz *J* = 4.3 Hz), 2.41 (d, 1H, 8$_{eq}$-H, *J* = 14.8 Hz), 2.48 (s, 3H, 9-C(O)Me), 2.95 (d, 1H, 10$_{ax}$-H, *J* = 18.8 Hz), 3.25 (d, 1H, 10$_{eq}$-H, *J* = 18.8 Hz), 3.35 (d, 1H, 4'-OH, *J* = 6.2 Hz), 3.66 (s, 3H, -CO_2Me), 3.83 (bd, 1H, 4'-H, *J* = 5.1 Hz), 4.08 (s, 3H, 4-OMe), 4.09 (d, 1H,Gluc5-H, *J* = 9.3 Hz), 4.28 (q, 1H, 5'-H, *J* = 6.6 Hz), 4.33 (bs, 1H, 3'-H), 4.63 (s, 1H, 9-OH), 4.86 (k, 1H, ArC*H*MeNH-, *J* = 7.4 Hz), 5.00-5.25 (m, 4H, Gluc2,3,4-H 7-H), 5.58 (d, 1H, 1'-H, *J* = 3.5 Hz), 5.65 (s, 1H, Gluc1-H, *J* = 8.3 Hz), 6.18 (d, 1H, ArCHMeN*H*-, *J* = 8.2 Hz), 6.57 (d, 1H, 3'-NH-, *J* = 8.8 Hz), 7.15-7.35 (m, 5H, Ar3,4,5,6-H), 7.38 (d, 1H, 3-H, *J* = 8.5), 7.78 (t, 1H, 2-H, *J* = 8.1 Hz), 8.03 (d, 1H, 1-H, *J* = 7.7 Hz), 13.29 (s, 1H, 11-OH), 14.00 (s, 1H, 6-OH).

N-2-(Daunorubicin-3'-N-carbonyl-α-methylbenzyl) O-β-D-glucuronyl carbamate sodium salt (**DAU-GB9**) (mixture of both diastereoisomers) from **60d** according to general procedure #6 in 95%, mp 195ºC (dec.), elem. anal.: calc. (found) for $C_{43}H_{45}N_2O_{19}\cdot 6\ H_2O$: C: 50.39 (49.87), H: 5.61 (5.05), N: 2.73 (2.75). MS (FAB$^+$) *m/z* = 940 ([M+1+Na]$^+$), 939 ([M+Na]$^+$), 918 ([M+1+H]$^+$), 917 ([M+H]$^+$). ^{1}H-NMR (100 MHz, $(CD_3)_2SO$) δ (ppm) = 1.15 and 1.18 (2d, 3H, 5'-Me, *J* = 6.5 and 6.4 Hz resp.), 1.29 (d, 3H, ArCH*Me*NH-, *J* = 5.7 Hz), 1.55-1.60 (m, 1H, 2'$_{eq}$-H), 1.99 (bt, 1H, 2'$_{ax}$-H, *J* = 11.8 Hz), 2.12 (dd, 1H, 8$_{ax}$-H, *J* = 13.7 Hz *J* = 5.2 Hz), 2.23 (dd, 1H, 8$_{eq}$-H, *J* = 12.4 *J* = 2.8 Hz), 2.29 (s, 3H, -C(O)Me), 2.95-3.20 (m, 6H, 10$_{eq}$-H 10$_{ax}$-H 4'-H Gluc2,3,4-H), 3.60 and 3.67 (2bs, 1H, 3'-H), 3.98 (s, 3H, 4-OMe), 4.10-4.30 (m, 2H, 5'-H Gluc5-H), 4.64 (bs, 1H, 4'-OH), 4.85 (t, 1H, ArCHMeN*H*-, *J* = 7.5 Hz), 4.90-5.30 (m, 5H, 7-H Gluc1-H Gluc2,3,4-OH), 5.28 (bs, 1H, 1'-H), 5.58 (s, 1H, 9-OH), 7.20-7.30 (m, 2H, 3'-NH- Ar3-H), 7.39 (t, 1H, Ar5-H, *J* = 6.4 Hz), 7.47 (t, 1H, Ar4-H, *J* = 8.0 Hz), 7.62 (bs, 1H, 3-H), 7.85-7.90 (m, 2H, 1,2-H), 7.99 and 8.06 (2d, 1H, Ar6-H, *J* = 7.7 and 8.2 Hz resp.), 14.05 (s, 1H, 6-OH).

Attempted synthesis of *N*-2-(daunorubicin-3'-*N*-carbonyl-α,α-dimethylbenzyl) *O*-β-D-glucuronyl carbamate sodium salt (DAU-GB10)

Homophthalic acid ω-allyl α-2-(trimethylsilyl)ethyl diester (**37**). 500 mg of **34**, 0.137 mL (1.05 equiv.) of 2-(trimethylsilyl)ethanol and a catalytic amount of DMAP were dissolved in CH_2Cl_2 and cooled to 0ºC. 490 mg (1.05 equiv.) of DCC were added and after 1 h, the reaction mixture was allowed to warm to ambient temperature and stirring was continued overnight. After that, the solid material was removed by filtration, the filtrate was diluted with *ca.* 100 mL of *n*-Hex and washed with 100 mL portions of a 5% aqueous solution of $KHSO_4$ (2x), saturated aqueous $NaHCO_3$ (2x) and brine. The solution was dried over Na_2SO_4 and evaporated. The crude diester was purified by column chromatography (SiO_2 *i*-Pr_2O) to yield 679 mg, 93% of **37** as a slightly yellowish oil, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 0.01 (s, 9H, $SiMe_3$), 1.01 (t, 2H, -OCH_2CH_2Si-, *J* = 8.6 Hz), 3.95 (s, 1H, ArCH_2-), 4.26 (t, 2H, -OCH_2CH_2Si-, *J* = 8.6 Hz), 4.48 (d, 2H, -OCH_2CH=CH_2, *J* = 5.6 Hz), 5.00-5.25 (m, 2H, =CH_2), 5.60-6.00 (m, 1H, -CH=), 7.05-7.40 (m, 3H, Ar3,4,5-H), 7.91 (dd, 1H, Ar6-H, *J* = 7.2 Hz *J* = 1.8 Hz).

α,α-Dimethylhomophthalic acid ω-allyl α-2-(trimethylsilyl)ethyl) diester (**38**) from **37** according to general procedure #7 in 36%, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 0.00 (s, 9H, $SiMe_3$), 1.04 (t, 2H, -OCH_2CH_2Si-, *J* = 8.7 Hz), 1.59 (s, 6H, -CMe_2-), 4.25 (t, 2H, -OCH_2CH_2Si-, *J* = 8.7 Hz), 4.48 (dt, 2H, -OCH_2CH=CH_2, *J* = 5.0 Hz *J* = 1.3 Hz), 4.95-5.20 (m, 2H, =CH_2), 5.60-6.00 (m, 1H, -CH=), 7.10-7.50 (m, 3H, Ar3,4,5-H), 7.81 (dd, 1H, Ar6-H, *J* = 7.0 Hz *J* = 1.3 Hz).

α,α-Dimethylhomophthalic acid α-2-(trimethylsilyl)ethyl ester (**40**) from **38** according to general procedure #3 in quantitative yield as an oil.

N-(3,3-Dimethylisoindolin-1-on) O-(methyl 2,3,4-tri-O-acetyl β-D-glucuronyl) carbamate (**62**) was unexpectedly obtained in 39% from **40** as an oil following general procedure #2. The glucuronyl donor was reacted with the *in situ* formed isocyanate at 95ºC, at lower temperatures no reaction took place. **62** was formed in a 1/1 mixture of the α- and β-diastereoisomers. The absence of β-diastereoselectivity is proposed to be due to the high reaction temperature. All double signals in the proton NMR were of α- and β-anomers. MS (FAB$^+$) *m/z* = 545 ([M+1+Na]$^+$), 544 ([M+Na]$^+$). ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.04 and 1.11

(2s, 3H, ArCMe_aMe$_b$-), 1.65 and 1.70 (2s, 3H, ArCMe$_a$$Me_b$-), 3.62 and 3.68 (2s, 3H, -CO_2Me), 4.23 (d, 0.5H, Gluc5-H$_\beta$, J = 9.0 Hz), 4.42 (d, 0.5H, Gluc5-H$_\alpha$ J = 10.5 Hz), 4.82 (dd, 0.5H, Gluc2-H$_\alpha$ J = 10.0 Hz J = 3.4 Hz), 4.95-5.65 (m, 3H, Gluc3,4-H 0.5Gluc2-H$_\beta$ 0.5Gluc1-H$_\beta$), 5.93 (d, 0.5H, Gluc1-H$_\alpha$, J = 3.0 Hz), 7.30-7.70 (m, 3H, Ar3,4,5-H), 7.81 (bd, 1H, Ar6-H, J = 7.4 Hz).

3.7 References and notes

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[23] Contrarily to the infeasible deprotection of **57**, it was possible to deprotect **55a** to **55c** because the starting material **55a** was much easier prepared than **57** and available in larger quantities. A large scale optimized procedure was performed to deprotect **55a**.

[24] **DAU-GB1** and **-GB5** (together with **DAU-GA1** (Ch. 2) and **DAU-GA3** (Ch. 4) and *β*-D-glucuronyl prodrugs prepared by others) were extensively characterized for use in ADEPT, see: Houba, P.H.J.; Leenders, R.G.G.; Boven, E.; Scheeren, J.W.; Pinedo, H.M. Haisma, *Biochem. Pharmacol.* **1996**, *52*, 455. In this paper, **DAU-GB5** is refered to as "GB6".

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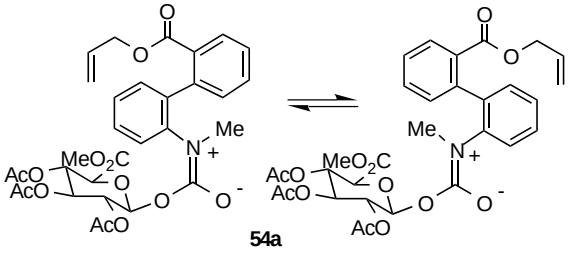
[28] Less than a stoichiometric amount of **9** was added because a fraction of the *in situ* formed isocyanate dimerizes. In case of **34** (scheme 3.8), 22 % of the symmetrical urea was isolated after completion of the reaction.

[29] In the ^{1}H-NMR spectrum of **50a** almost all signals are double, indicating the existance of two rotameric forms of the compound caused by restricted rotation around the Ar-Ar bond (see experimental part of **DAU-GB4**). In case of **54a**, additional to this restricted rotation, the existence of two tautomers of the carbamate also causes double signals or broadening of signals in the ^{1}H-NMR spectrum. This was also observed for **54c** and **DAU-Me-GB4**.



54a

4 Anthracycline-Spacer-β-D-Glucuronyl Carbamates as Prodrugs [1]

Spacer Expulsion by 1,6-Elimination

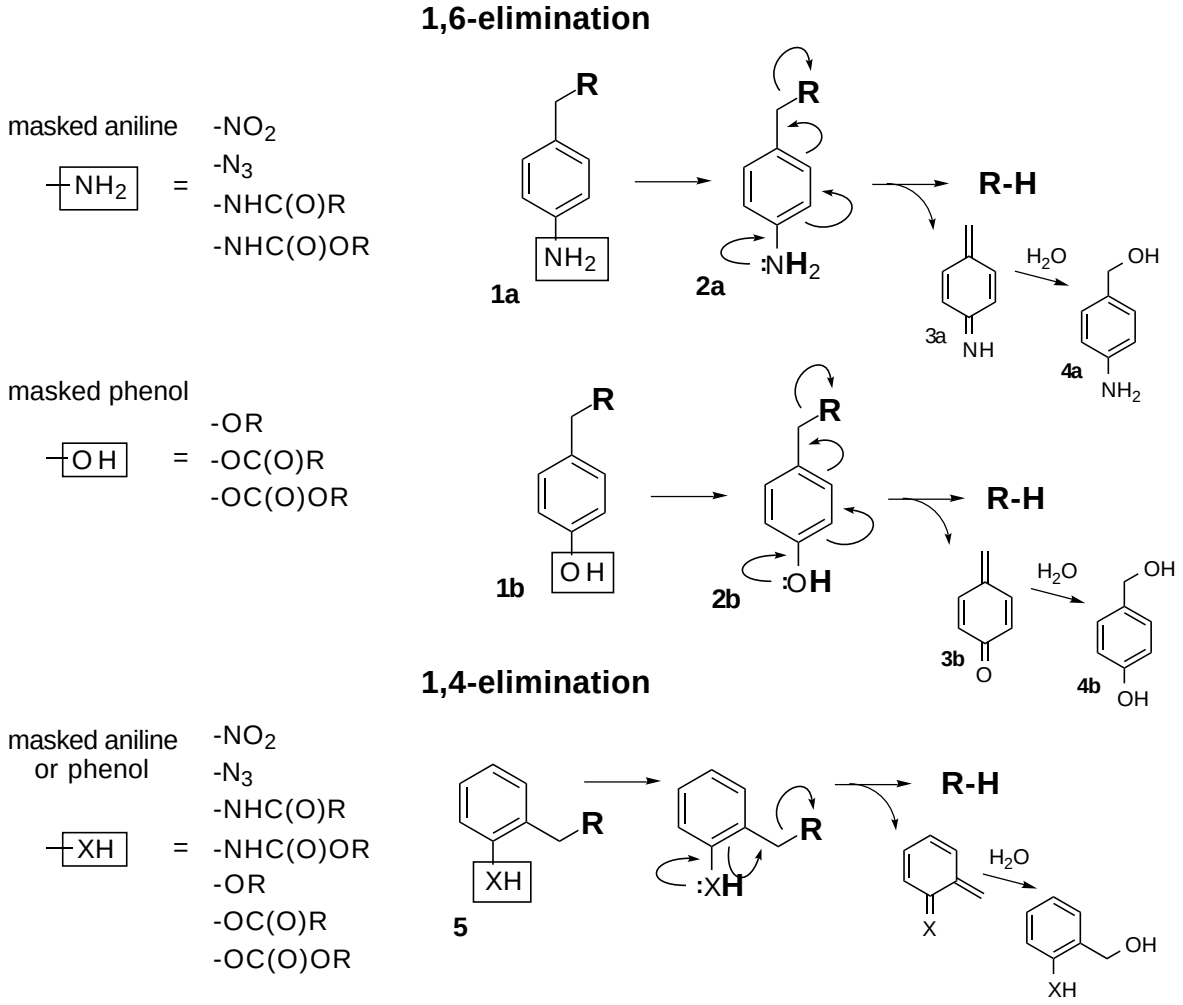
4.1 Introduction

4.1.1 General

Improvement of β-glucuronidase mediated rates of hydrolysis of prodrugs by incorporation of a spacer moiety between daunorubicin and the β-D-glucuronyl carbamate specifier is apparent, see chapter 3. In this respect, one of these drug-spacer-β-D-glucuronyl carbamate prodrugs (**DAU-GB8**, chapter 3) was hydrolyzed up to 1000-fold faster by β-glucuronidase compared to a non-spaced prodrug (**DAU-GA1**, chapter 2). Disappointingly, elimination of the spacer from the drug-spacer molecule did not occur after the triggering step. The potential of a β-D-glucuronyl carbamate as a readily cleavable specifier, however, is appreciated. Therefore, in the present chapter, other self-immolating spacers in combination with a β-D-glucuronyl carbamate specifier are investigated.

4.1.2 The 1,4- and 1,6-elimination

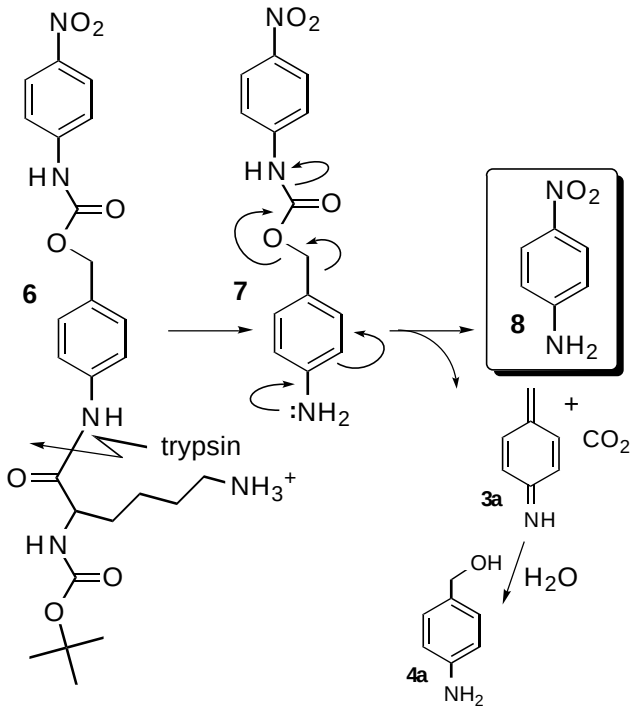
The 1,4- and 1,6-elimination[2] of R-H from benzylic systems *ortho* or *para* substituted with the strong electron-releasing amino- or hydroxy-group (**2a** and **2b**, respectively, scheme 4.1) has long been known and reviewed in the early nineties by Wakselman[3]. In order to trigger the 1,6-elimination process, the strongly electron-donating amino- or hydroxy-group in **2a** and **2b**, respectively, is generated from a less electron-donating masked amino- or hydroxy-functionality, **1a** and **1b**, respectively. The 1,6-elimination reaction is a fast process and is dependent on the nature of the leaving group -R. Literature examples of masked amine- or hydroxy-functionalities are mainly found in protective group chemistry. These include amide-[4], nitro-[5] and azido-groups[6], and ester-[7] and carbonate-[7] groups, respectively, which upon demasking to an amino- or hydroxy-group trigger the 1,6-elimination process. During this elimination, the compounds R-H and **3a** and **3b** are formed, which, in aqueous media, add water resulting in *p*-amino- and *p*-hydroxy-benzyl alcohol **4a** and **4b**, respectively (scheme 4.1).



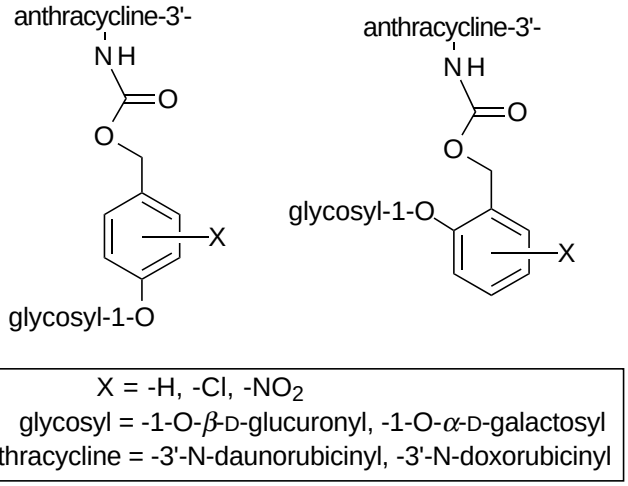
Scheme 4.1 *1,4- And 1,6-elimination of hydroxy- and amino-benzyl systems.*

In this regard, the use of a natural amino acid amide as a masked aminobenzyl spacer in prodrug preparation was proposed by the group of Katzenellenbogen[8] (scheme 4.2). This model system **6** rapidly releases the chromophore *p*-nitroaniline **8** (the model for a drug in this system) after hydrolysis by trypsin of the N^{α}-Boc-Lys specifier group. The half-life of decay of the intermediate **7** was estimated to be *ca* 1.5 min. Furthermore, during the course the research described in this thesis, a doxorubicin-aminobenzyl spacer-α-D-galactosyl prodrug, compound **9** chart 4.2, was reported[9] employing the amino benzyl spacer. Detailed characteristics of this compound as a prodrug in ADEPT, however, were not described probably because of a too high toxicity of the prodrug[10]. Additionally, collaborating German and French scientists published a series of papers

and communications on *para* and *ortho* -hydroxybenzyl spacer-glycoside anthracycline prodrugs [11]. These prodrugs are depicted in chart 4.1. Electron-withdrawing substituents on the spacer of the prodrugs in chart 4.1 facilitate enzymatic hydrolysis. For unsubstituted prodrugs (X = -H in chart 4.1), however, spacer elimination after enzymatic hydrolysis of the glycosyl specifier did not take place [11c]. This was ascribed to the insufficient acidity of the phenolic function of the spacer. Fast elimination of the spacer was observed for prodrugs with a nitro group on the spacer (X = -NO_2). On account of this, doxorubicin prodrug **10** (chart 4.2), was selected by Bosslet and coworkers for extensive preclinical *in vivo* studies in nude mice [12] and was found to possess agreeable though not ideal characteristics as a prodrug in ADEPT (e.g. prodrug toxicity, enzyme activation rate). The enzymatic activation rate of prodrug **10** is still not optimal even though one of the strongest electron-withdrawing groups (the nitro group) is present on the aromatic ring of the spacer.



Scheme 4.2 *Application of self-immolating p-aminobenzyl spacer in prodrug design.*



X = -H, -Cl, -NO_2
glycosyl = -1-O-β-D-glucuronyl, -1-O-α-D-galactosyl
anthracycline = -3'-N-daunorubicinyl, -3'-N-doxorubicinyl

Chart 4.1 *p- And o-hydroxybenzyl spaced prodrugs of anthracyclines reported in the literature [11].*

Additionally, the excretion half-life of prodrug **10** from the serum of mice is 0.4 h precluding efficient treatment. As already stated in sections 2.2.2 and 4.1.1, a β-D-glucuronyl carbamate group is a promising specifier because its ease of hydrolysis

by β-glucuronidase. In this view, prodrugs of anthracyclines containing an aminobenzyl spaced β-glycosyl carbamate specifier group and their features for use in ADEPT and/or monotherapy[29] were investigated.

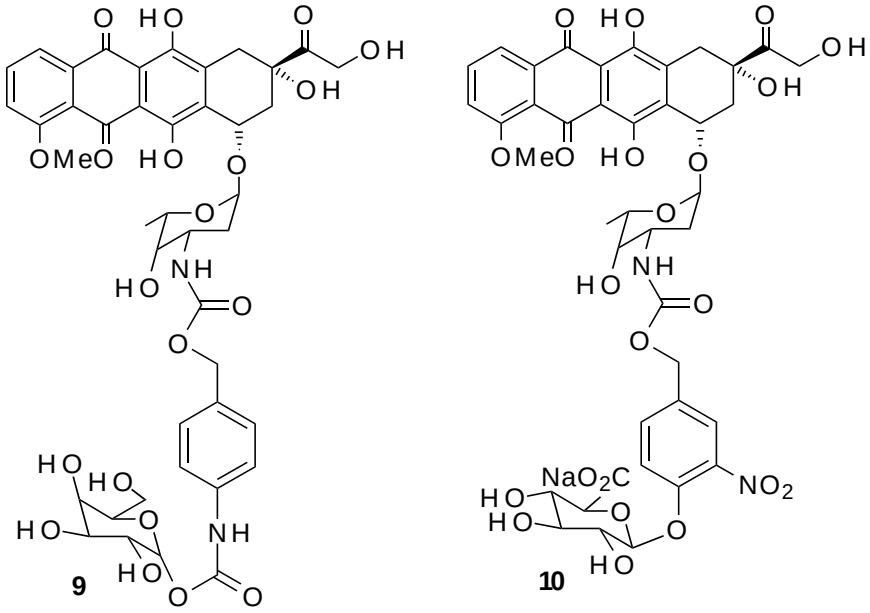
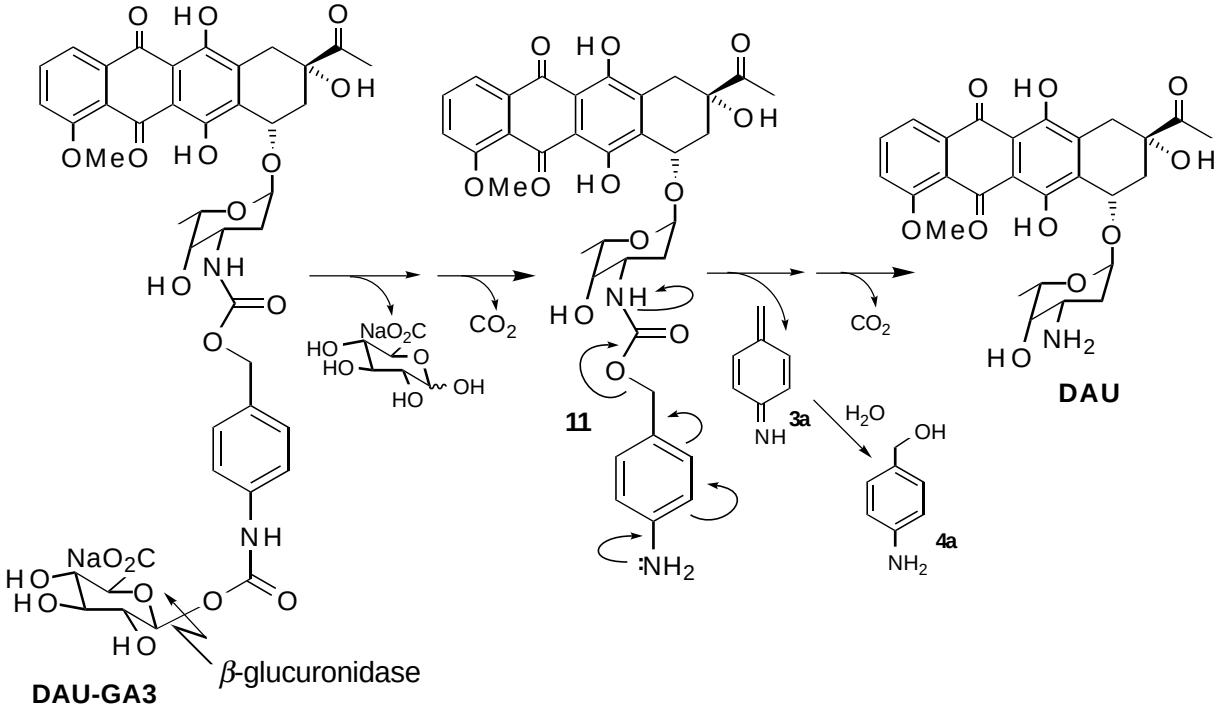


Chart 4.2 *Two prodrugs reported in the literature.*

4.2 Target compounds

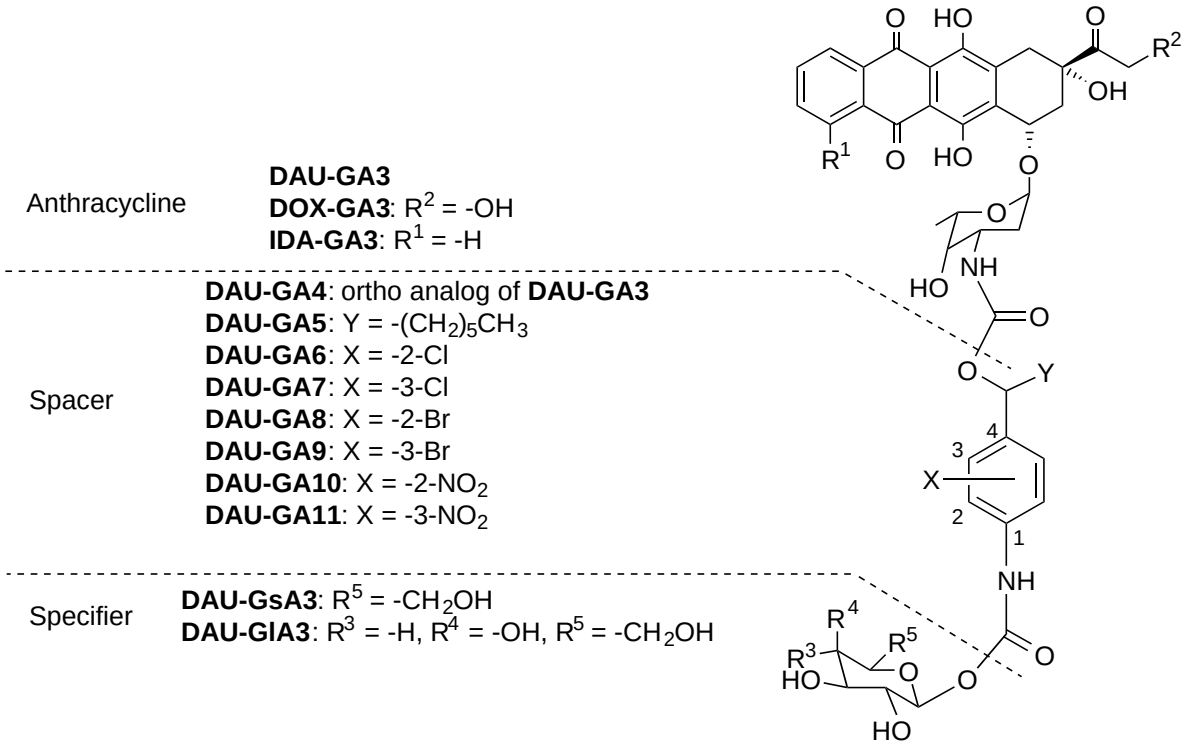


Scheme 4.3 ***DAU-GA3*** *And its activation pathway to the free drug.*

We focused exclusively on masked *para*- (and *ortho*) aminobenzyl systems **1a** (scheme 4.1) for utilization as a self-immolating spacer to allow the use of a *β*-D-glucuronyl carbamate as a masked amine. In a first study, *β*-D-glucuronyl carbamate based prodrug **DAU-GA3**[13] (scheme 4.3) was synthesized. Because daunorubicin is the easiest available anthracycline, it was used for the initial prodrug preparation. Prodrug **DAU-GA3** will be activated to daunorubicin upon contact with *β*-glucuronidase according to scheme 4.3. Hydrolysis of the *β*-D-glucuronyl specifier and loss of a molecule of CO_2 results in drug-spacer molecule **11**. Compound **11** spontaneously undergoes 1,6-elimination and concomitant loss of a second molecule of CO_2 to give daunorubicin and iminoquinone methide **3a** which reacts with water to provide *p*-aminobenzyl alcohol **4a**. Preliminary test results[14] of **DAU-GA3** revealed the following: *i.* The half life of activation to daunorubicin by *β*-glucuronidase under standard conditions (100 μM prodrug, 1 μg/mL enzyme, pH = 6.8, 37°C) was 135 min. *ii.* The IC50 value of **DAU-GA3** was about 100-fold higher compared to the parent drug and *iii.* **DAU-GA3** was cleared from the serum of a mouse in a half life of 20 min. Optimal prodrugs should combine fast enzymatic hydrolysis with relatively slow clearance from the circulation (the 100-fold reduced toxicity of the prodrug was found to be satisfactory). It was hypothesized that rates of hydrolysis for prodrug activation and rates of clearance of the prodrug from the blood stream could be influenced by substituents on the spacer and variation of the specifier. Accordingly, variations of prodrug **DAU-GA3** were designed (chart 4.3) possessing substituents on the spacer, or having a different specifier group. In addition, prodrugs of doxorubicin and idarubicin (see chart 1.3) were synthesized and evaluated. The series of target compounds thus

derived from **DAU-GA3** are collected in chart 4.3. The planned molecular variations are summarized as follows:

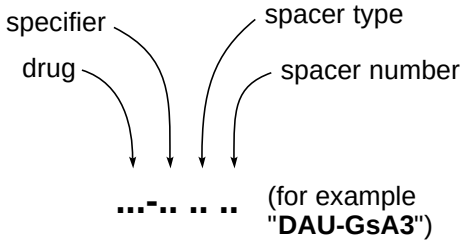
In all prodrugs,
R^1 = -OMe, R^2 = -H, R^3 = -OH, R^4 = -H, R^5 = -CO_2Na, X = -H, Y = -H
unless indicated otherwise.



nomenclature of glycosylated prodrugs:

anthracycline: **DAU** = daunorubicin
DOX = doxorubicin
IDA = idarubicin

specifier: **G** = β-D-glucuronyl carbamate
Gs = β-D-glucosyl carbamate
Gl = β-D-galactosyl carbamate



spacer type: **A** =spacer expulsion by 1,4- or 1,6-elimination (Ch. 4) or -CO_2 spacer (Ch. 2)
B = spacer expulsion by cyclization (Ch. 3)

spacer number: numbering of prodrugs of same type

Chart 4.3 *Target compounds derived from **DAU-GA3**.*

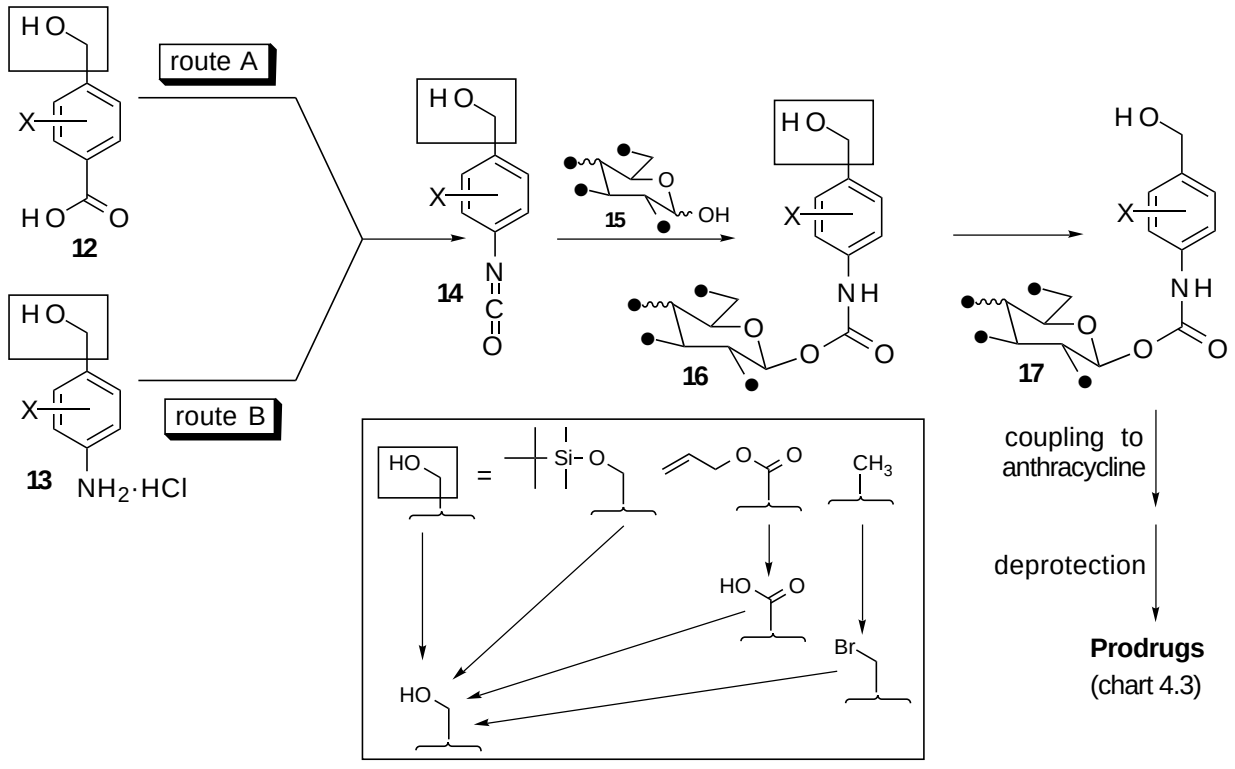
The specifier: β-D-glucoside and β-D-galactoside derivatives **DAU-GsA3** and **DAU-GlA3**, respectively, are expected to be activated by human β-glucosidase and human β-galactosidase, respectively. These prodrugs possess a reduced polarity which might retard rapid clearance from the blood.

The spacer: The *ortho* analog of **DAU-GA3**, compound **DAU-GA4**, as well as prodrugs **DAU-GA6 - 11** containing a chlorine, bromine or nitro substituent on the aromatic ring of the spacer will be synthesized. Apart from being electron withdrawing and, therefore, facilitating the enzymatic hydrolysis of the *β*-D-glucuronyl group, chloro- and bromo-aryl groups are known to have a high serum albumin binding potency[15] which might prevent rapid prodrug excretion. Furthermore, prodrug **DAU-GA5** having a lipophylic C_6-tail on the benzylic carbon of the spacer was designed in order to study excretion pharmacokinetics.
The anthracycline: Doxorubicin and idarubicin analogs **DOX-GA3** and **IDA-GA3**, respectively, are included in the series of targets, especially because doxorubicin is clinically more relevant than daunorubicin.
It should be noted that **DAU-GlA3** is analogous to compoud **9** (chart 4.2) except that the anthracycline is not doxorubicin but daunorubicin and that the specifier in **DAU-G1A3** is a *β*-galactosyl carbamate whereas in **9** the specifier is an *α*-galactosyl carbamate. Furthermore, all compound in chart 4.1 contain a *β*-D-glucuronyl specifier group but lack the connecting carbamate function between spacer and specifier which is present in all prodrugs in chart 4.3.

4.3 Synthesis of prodrugs

4.3.1 Strategy

The designed prodrugs presented in chart 4.3 can be prepared by coupling the benzyl alcohol group of pro-moieties **17** to the anthracycline 3'-amino group.



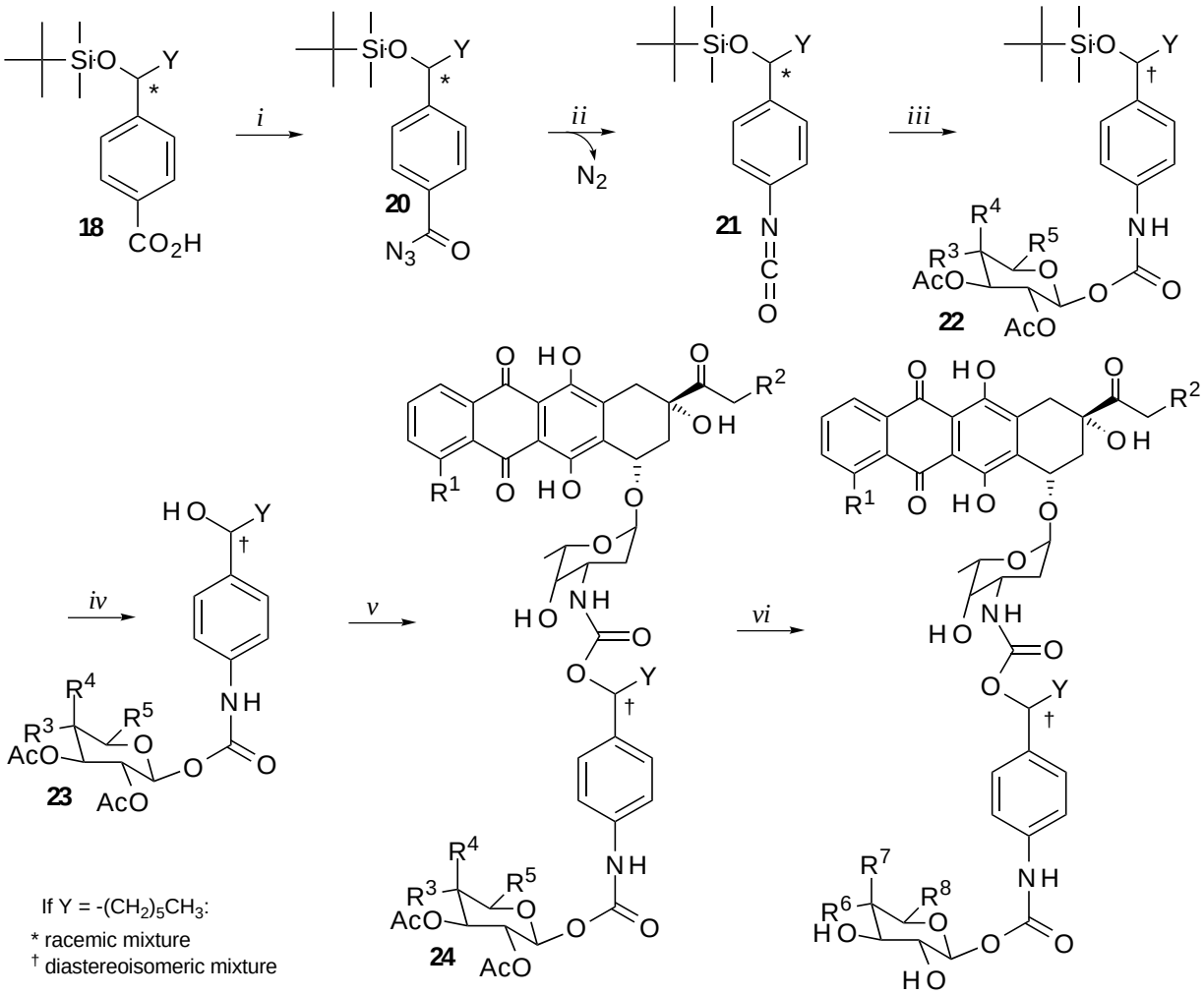
Scheme 4.4 *General synthetic pathways leading to pro-moiety **17**.*

For the preparation of compound **17** *via* masked intermediates **16**, in routes A and B (scheme 4.4), introduction of a *β*-glycosyl carbamate group to the spacer can be accomplished by the addition reaction of the anomerically unprotected glycosyl donor **15** to spacer isocyanate **14**. As outlined in section 5.1, this isocyanate addition reaction when performed in toluene and in the presence of triethylamine as the catalyst, leads to a very high *β*-diastereoselectivity[16]. Depending on the availability of the starting material for **12** and **13**, the masked hydroxymethyl unit was either a protected hydroxymethyl group an ester or a methyl group. These units can be converted into a hydroxymethyl group by deprotection, reduction and oxidation, respectively. Following these pathways, pro-moieties **17** can either be prepared from carboxylic acids **12**, aniline-hydrochlorides **13** or isocyanates **14**.

4.3.2 Synthesis of prodrugs *via* route A

The rearrangement of acyl azides to isocyanates can be employed in the synthesis of prodrugs to generate isocyanates **21** from carboxylic acids **18** (scheme 4.5). Using diphenyl phosphoryl azide **19**[17], spacer acyl azides **20**[18] were formed from carboxylic acids **18**. Upon heating, acyl azides **20** rearranged to isocyanates **21**. After these isocyanates had been formed, the mixture was cooled to ambient temperature and the anomerically unprotected glycosyl donor **15** was added. Following this protocol, *β*-D-glycosyl carbamates **22** were obtained in a one-pot procedure from carboxylic acids **18** in good to excellent yields and in more than 95% *β*-diastereoselectivity.

Attempts to remove the *tert.*-butyldimethylsilyl group from **22** using TBAF resulted in multiple product formation, probably due to removal of the acetyl protection groups by the basic TBAF. Alternatively, selective removal of the silyl protection group of intermediates **22** using acetic acid/water/THF was successfully achieved in almost quantitative yield. The benzyl alcohol group of **23** was coupled to daunorubicin, doxorubicin or idarubicin making use of di-*N*,*N*'-succinimidyl carbonate (**25**)[19]. Deprotection of the carbohydrate specifier of **24** was accomplished using lithium hydroxide in MeOH/H_2O/THF at 0ºC. After neutralization of the basic solution with ion exchange resin, the polar prodrugs were purified on a reversed phase-C_{18} column and lyophilized to yield the pure prodrugs as red fluffy solids. The most troublesome step in the preparation of prodrug **DOX-GA3** was the deprotection of the glucuronyl moiety of the protected precursor **24(DOX-GA3)**[20]. A maximum yield of 37% was obtained due to the base lability of doxorubicin, whereas for daunorubicin prodrug **DAU-GA3** deprotection was readily accomplished in 81%.



	R^1	R^2	R^3	R^4	R^5	R^6	R^7	R^8	Y
DAU-GA3	-OMe	-H	-OAc	-H	$-CO_2Me$	-OH	-H	$-CO_2Na$	-H
DOX-GA3	-OMe	-OH	-OAc	-H	$-CO_2Me$	-OH	-H	$-CO_2Na$	-H
IDA-GA3	-H	-H	-OAc	-H	$-CO_2Me$	-OH	-H	$-CO_2Na$	-H
DAU-GsA3	-OMe	-H	-OAc	-H	$-CH_2OAc$	-OH	-H	$-CH_2OH$	-H
DAU-GlA3	-OMe	-H	-H	-OAc	$-CH_2OAc$	-H	-OH	$-CH_2OH$	-H
DAU-GA4[*]	-OMe	-H	-OAc	-H	$-CO_2Me$	-OH	-H	$-CO_2Na$	-H
DAU-GA5	-OMe	-H	-OAc	-H	$-CO_2Me$	-OH	-H	$-CO_2Na$	$-(CH_2)_5CH_3$

[*] **DAU-GA4** is the *ortho* analog of **DAU-GA3**

Scheme 4.5 *Synthesis of prodrugs with unsubstituted spacer aromatic ring.*

The ortho substituted prodrug **DAU-GA4** (chart 4.4) was synthesized analogously to **DAU-GA3** (scheme 4.5) starting from the silyl-protected *o*-hydroxymethyl benzoic acid **27** (chart 4.4).

DAU-GA5 was synthesized according to scheme 4.5, with Y = -*n*-hexyl . The starting material **18** (Y = -*n*-hexyl), was obtained by following scheme 4.6. The secondary benzyl alcohol group of pro-moiety **24** was coupled to daunorubicin using the more reactive *N*-succinimidyl chloroformate [21] (**26**, scheme 4.5).

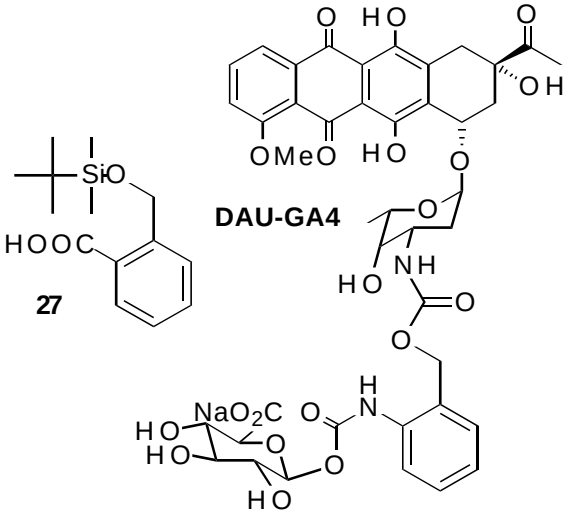
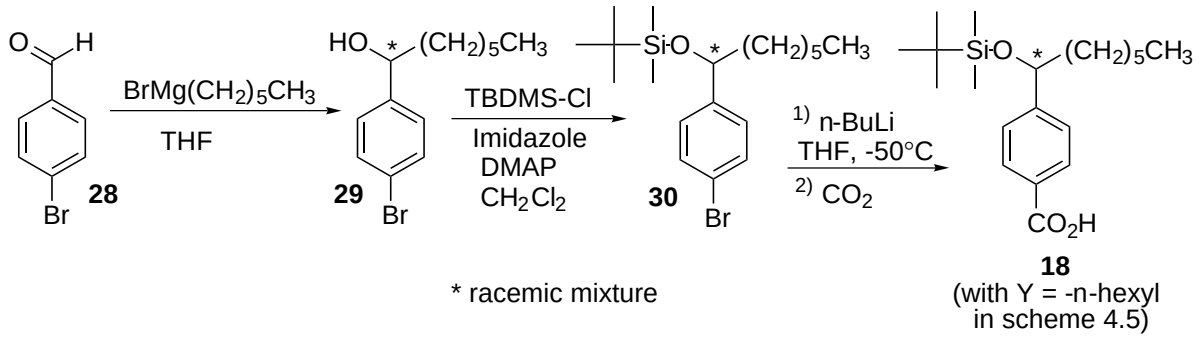


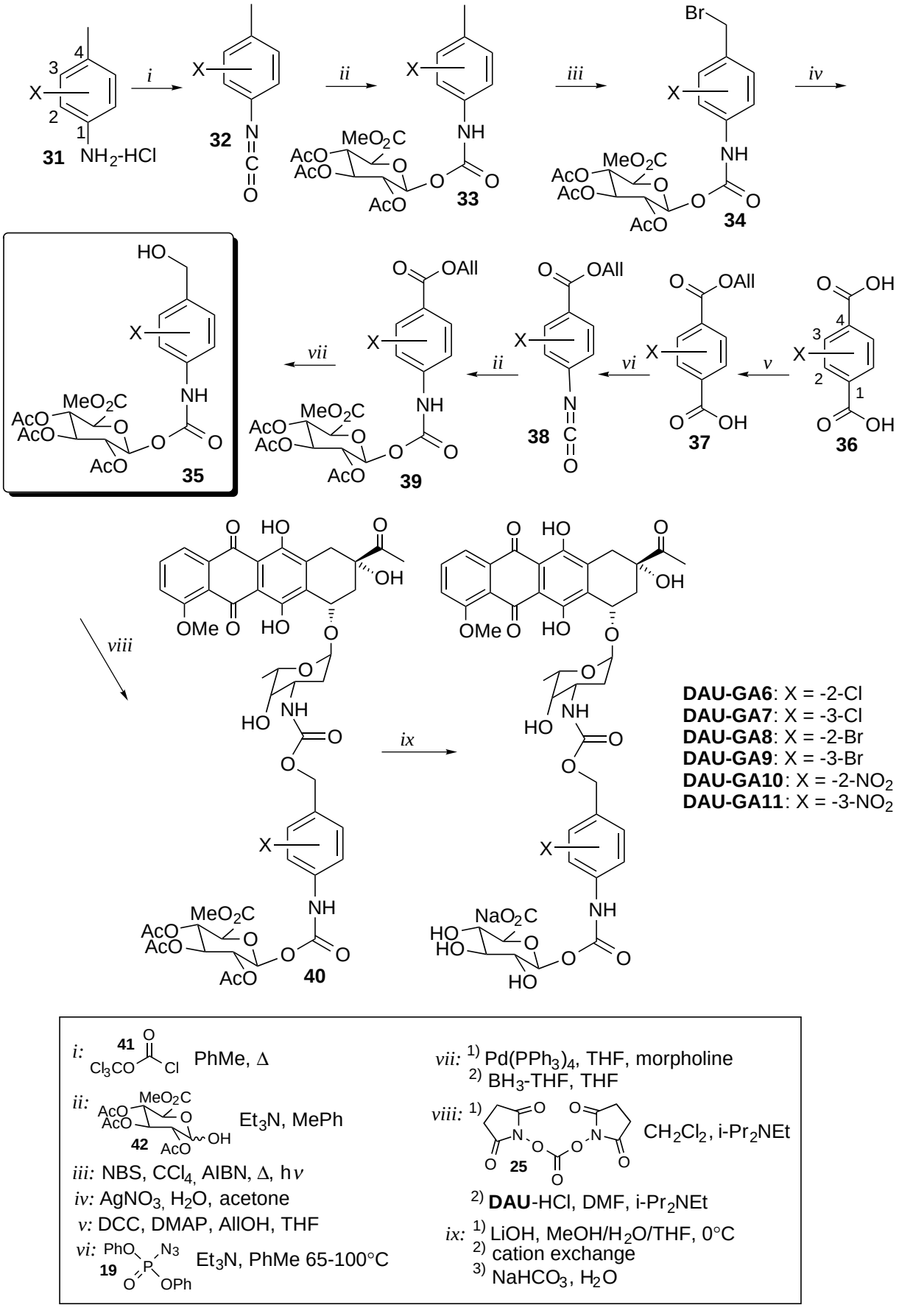
Chart 4.4 *Ortho analog of* ***DAU-GA3****.*



Scheme 4.6 *Preparation of* ***18****.*

4.3.3 Preparation of prodrugs containing electron-withdrawing substituents on the spacer.

Pro-moieties for prodrugs **DAU-GA6** - **-GA11** containing an additional chlorine, bromine or nitro group on the spacer were synthesized according to scheme 4.7. Depending on the availability of the starting material, the pro-moieties were prepared either from substituted anilines **31**[22], toluic isocyanates **32** or terephthalic acid derivatives **36**. The spacer-2-chloro and spacer-2-bromo substituted pro-moieties for prodrugs **DAU-GA6** and **DAU-GA8**, respectively, were prepared in moderate yield starting from 4-methyl-2-chloroaniline-HCl and 4-methyl-2-bromoaniline-HCl (**31**, X = -2-Cl and -2-Br, respectively) using diphosgene (**41**)[23] to make the respective isocyanates **32**. After the addition of glucuronyl donor **42** to isocyanates **32**, the 4-Me group *β*-D-glucuronyl carbamates **33** was brominated. The crude bromides **34** were hydrolyzed using silver nitrate in acetone/water yielding pro-moieties **35** (X = -2-Cl) and **35** (X = -2-Br) in 50 - 80% yield.

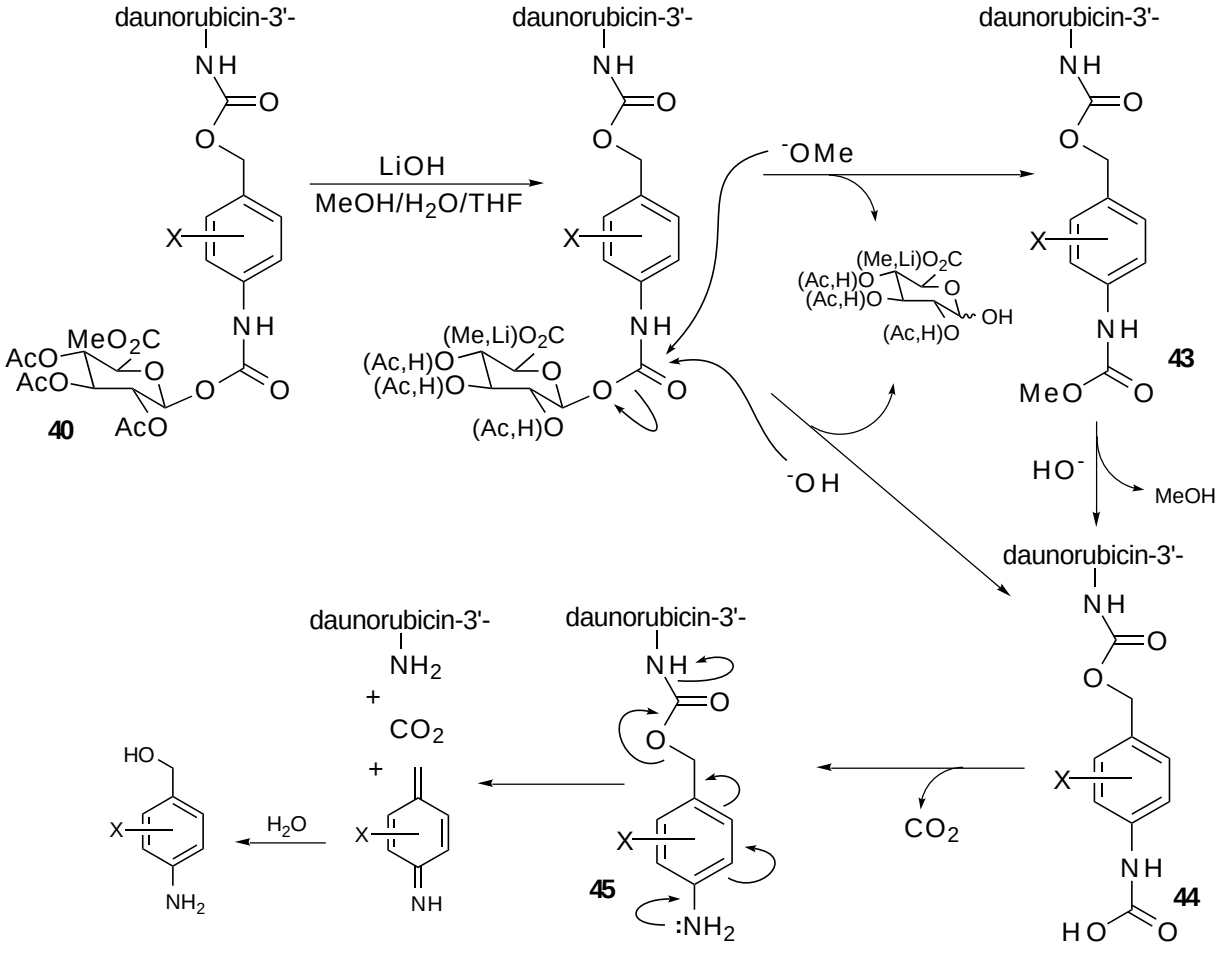


Scheme 4.7 *Synthesis of substituted prodrugs [13].*

The pro-moieties for prodrugs **DAU-GA7**, **-GA10** and **-GA11** were analogously synthesized starting from the respective commercially available isocyanates **32** (X = -3-Cl, -2-NO_2 and -3-NO_2, respectively). As 3-bromo-4-methyl isocyanate **32** (X = -3-Br) was not commercially available, the corresponding pro-moiety **35** (X = -3-Br) was synthesized from bromoterephthalic acid (**36**, X = -Br) by esterification of one carboxylic acid group and selective crystallization of the C(4) allyl ester bromo terephthalic acid derivative **37** (X = -3-Br). Isocyanate **38** (X = -3-Br) was generated from carboxylic acid **37** (X = -3-Br) employing the Curtius rearrangement. This isocyanate was further reacted with anomerically unprotected glucuronate **42** to give **39** (X = -3-Br). Removal of the allyl protection group and reduction of the resulting carboxylic acid to a benzyl alcohol group using BH_3-THF[24] resulted in pro-moiety **35** (X = -3-Br) in excellent yield.

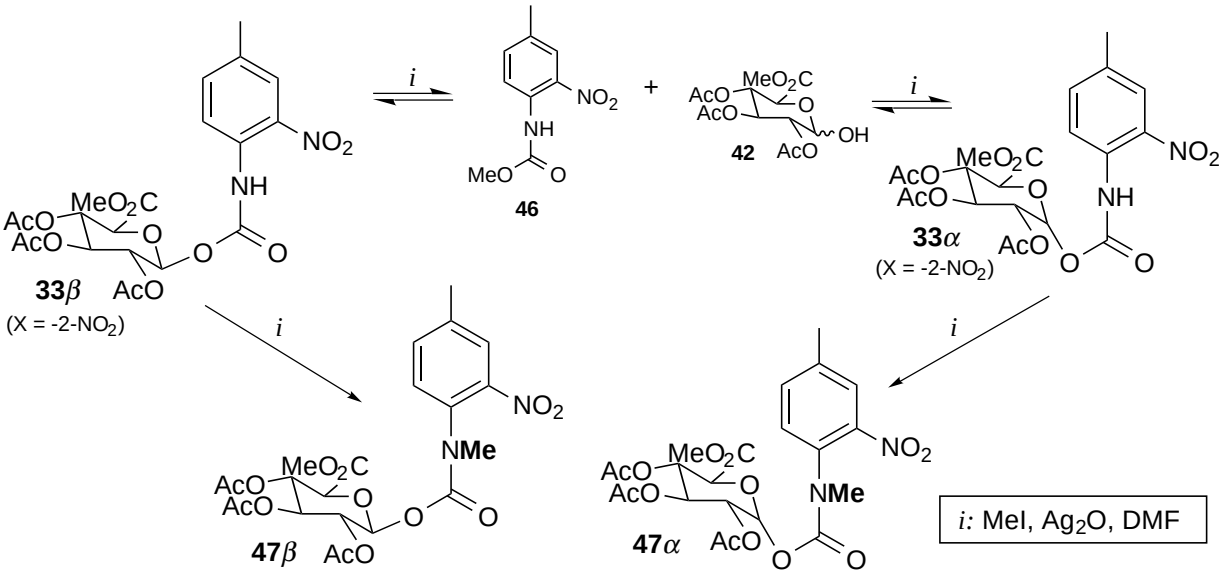
The thus obtained six pro-moieties **35** were coupled to daunorubicin using di-*N,N'*-succinimidyl carbonate (**25**)[19] to give protected prodrugs **40**.

The procedure to hydrolyze the three acetyl groups and the methyl ester of the glucuronyl fragment of the protected prodrugs, proved to be the most troublesome step. Deprotection of the prodrug precursors **40** having the electronegative substituent on the 2-position of the aromatic ring led to extensive decomposition products. In case of both **DAU-GA6** and **-GA8** (X = -2-Cl and -2-Br, respectively), about 35% yield was attained.



Scheme 4.8 *Decomposition pathways during deprotection of protected prodrugs* ***40****.*

Deprotection of **40** (X = -2-NO_2) did not led to the desired prodrug **DAU-GA10**, but in nearly quantitative yield methyl carbamate **43** (X = -2-NO_2, scheme 4.8) was formed. Deprotection of the prodrug precursors **40** having the electronegative substituent on the 3-position of the aromatic ring was accomplished in satisfactory yields in case of **DAU-GA7** (67%) and **DAU-GA9** (50%). **DAU-GA11**, however, could not be obtained pure. Deprotection of **40** (X = -3-NO_2) led to extensive decomposition and multiple side-product formation and only a small amount of impure **DAU-GA11** was obtained.
The problematic base mediated demasking of the protected prodrugs **40** is very likely due to hydrolysis of the spacer carbamate group leading to product **44** (scheme 4.8) which further decomposes to daunorubicin. In the case of **40** (X = -2-NO_2) formation of methyl carbamate **43** (scheme 4.8) was observed.
To be able to prepare a prodrug containing a 2-nitro group on the spacer, it was anticipated to methylate the spacer nitrogen atom in order to prevent hydrolysis of the *β*-glucuronyl carbamate during base mediated deprotection of the prodrug as depicted in scheme 4.8. In this respect, when *N*-(2-nitro-4-methylphenyl) *O*-*β*-D-glucuronyl carbamate **33***β* (X = -2-NO_2, scheme 4.9) was treated with silver oxide and methyl iodide in DMF[25], the nitrogen atom was methylated and yielded the *N*-methyl derivative **47***β*. Along with the *N*-methylated *β*-product, the *N*-methylated *α*-product **47***α* was formed (reaction at 20°C, ratio of **47***α*/ *β* = 1/3, at 0°C no *α*-product was formed). This anomerization can be envisioned when methyl carbamate **46** and glucuronate **42** are formed in the reaction medium leading to both the *α*- and the *β*-anomer after recurring addition reaction[26] .



Scheme 4.9 *N-Methylation of β-D-glucuronyl carbamate **33** (X = -2-NO_2).*

Following scheme 4.7, starting from **47***β*-instead of **33**, the spacer-*N*-Me analog of prodrug **DAU-GA11** could not be obtained. Bromination at the 4-Me group of **47***β* and subsequent hydrolysis of the resulting bromide using silver oxide, led to multiple product formation. The corresponding *N*-Me hydroxymethyl derivative **35** (X = -2-NO_2)

was obtained in only minor amounts and attempts to couple this compound to daunorubicin were not undertaken.

4.4 Evaluation of all synthesized prodrugs for application in ADEPT

4.4.1 Cytotoxicities

The *in vitro* cytotoxicities of all prodrugs prepared in section 4.3 were determined on OVCAR-3 cells and compared to IC50 values of the respective parent drugs (table 4.1). In these *in vitro* experiments, all prodrugs displayed an antiproliferative effect that was at least 10-fold lower than their respective parent drug. In addition, in *in vivo* experiments, maximum tolerated doses (MTD) of daunorubicin prodrug **DAU-GA3** and doxorubicin prodrug **DOX-GA3** in nude mice bearing OVCAR-3 implants were 30 to 50 times higher (table 4.1) than the MTD of daunorubicin and doxorubicin, respectively. These findings indicate that these prodrugs possess a strongly decreased cytotoxicity in comparison to the parent drugs and are much better tolerated by a test animal, which is an essential feature for a prodrug. The large difference in the antiproliferative effects of prodrugs in *in vitro* and *in vivo* experiments can presumably be attributed to the rapid clearance from the circulation in the *in vivo* experiments. In *in vitro* experiments, *β*-glucuronidase liberated by defective cells activated the prodrug to some extent so that the free drug was generated, higher toxicities therefore were found.

Compound	IC50 (μM)[#]	Activation $t_{1/2}$(min)	MTD [¶] (mg/kg)
DAU-GA3	10	135 [*]	250
DOX-GA3	10	170 [*]	500
IDA-GA3	1	120 [*]	n.d.
DAU-GsA3	1.5	300 [‡]	n.d.
DAU-GlA3	2.5	400 [‡]	n.d.
DAU-GA4	11	125 [*]	n.d.
DAU-GA5	n.d.	950 [*]	n.d.
DAU-GA6	n.d.	150 [*]	n.d.
DAU-GA7	8.5	90 [*]	n.d.
DAU-GA8	n.d.	150 [*]	n.d.
DAU-GA9	n.d.	170 [*]	n.d.
DAU-GA11	n.d.	75 [*,†]	n.d.
daunorubicin	0.1	-	10
doxorubicin	0.05	-	8
idarubicin	0.01	-	

n.d.: not determined.
[#] Inhibition concentration on OVCAR-3 cells.
[*] 100 μM Prodrug, 0.03 U/mL human *β*-glucuronidase, pH = 6.8, 37°C.
[‡] 100 μM Prodrug, 0.3 U/mL *β*-galactosidase [28], pH = 6.8, 37°C.
[†] Hydrolysis to drug-spacer molecule, no conversion to daunorubicin.
[¶] Maximal tolerated dose in nude mice.

Table 4.1 *Cytotoxicities, enzymatic activation rates and MTD of prodrugs.*

In *in vivo* experiments, less premature prodrug activation will occur because β-glucuronidase levels in blood are very low[27] due to the excretion of the highly polar β-glucuronidase enzyme.

4.4.2 Pharmacokinetics

Disappointingly, the cellular accumulation and clearance from the circulation of nude mice of the glucosyl and galactosyl based prodrugs **DAU-GsA3** and **DAU-GlA3**, and of the more lipophylic prodrug **DAU-GA5**, was similar to these characteristics of **DAU-GA3**. Contrastingly, their water solubility compared with **DAU-GA3** was unfavourable. The *in vitro* serum protein binding of the halogen containing prodrugs **DAU-GA6** - **-GA9** was found to be higher than that of **DAU-GA3**. In contrast, elimination of these prodrugs from the blood stream of mice was comparable to that of **DAU-GA3**. The serum protein binding haloarylated drug derivatives described in the literature [15], possess a relatively small molecular mass (M = ± 250 g/mol). The halogenated prodrugs **DAU-GA6** - **-GA9** have a molecular mass of approximately 1000 g/mol. Probably the serum binding capacity of the halogenated prodrugs is too small to retain the prodrug in the bloodstream.

4.4.3 Enzymatic prodrug activation rates

For determination of the enzymatic activation rates, the prodrugs were incubated with the matching enzyme[28]. Incubation of **DAU-GA3** with human β-glucuronidase results in complete activation of the prodrug and on HPLC, no intermediate drug-spacer molecule was detected [14]. All prodrugs in the present chapter were quantitatively activated to the parent drug except the spacer-3-nitro substituted prodrug **DAU-GA11**. When the β-glucuronidase mediated activation of **DAU-GA11** was examined on HPLC the prodrug disappeared with a half-live of 75 min. Instead of daunorubicin, however, a peak of an unknown compound was detected which was assumed to be the drug-spacer molecule. Probably, electron density in the spacer aromatic ring is too strongly diminished by the presence of the nitro group for the 1,6-elimination process to take place.

Prodrugs of daunorubicin, doxorubicin and idarubicin containing the *para* or *ortho* glucuronyl carbamate pro-moiety were activated by β-glucuronidase at approximately the same rate. Half-lives between 2 and 3 hours were recorded (table 4.1). The glucosyl and galactosyl based prodrugs, however, were activated at a two-fold reduced rate compared with the glucuronyl based prodrugs. From the derivatives having an electron-withdrawing group on the spacer, only **DAU-GA7** displayed an accelerated activation rate as can be deduced from table 4.1.

4.4.4 Conclusions

As can be concluded from the data presented in the preceeding sections, the β-glucuronyl-based prodrugs of daunorubicin and doxorubicin, **DAU-GA3** and **DOX-GA3**, respectively, are acceptable prodrugs in selective chemotherapy (ADEPT). In monotherapy experiments (in which the mAb-enzyme conjugate is *not* given prior to prodrug administration) favorable selective toxicities of these prodrugs for target tissue

were observed, caused by endogenous β-glucuronidase liberated from necrotic tumor areas[29]. For more detailed studies using these prodrugs in both ADEPT and monotherapy experiments, see chapter 6. **IDA-GA3** was only studied in preliminary experiments.
Until now, structural variation of the spacer moiety of the prodrugs did not significantly improve prodrug characteristics, such as the rate of enzymatic hydrolysis and retention in the circulation of the prodrug.

4.5 Experimental Part

4.5.1 Biological evaluation

Cytotoxicities

The cytotoxicities of daunorubicin, doxorubicin, idarubicin and of all ten prodrugs on OVCAR-3 cells were determined by measuring cell growth with a protein dye stain[30]. Cells were harvested with 0.25% trypsin and 0.2% EDTA in PBS to obtain a single cell suspension and seeded in 96-wells tissue culture plates (2 x 10^6 cells/mL 10 μL/well, 3 wells per concentration). Drug or prodrug was added (10 μL/well) at different concentrations with a range of 3 or more logs. After incubation for 24 h, 200 μL of culture medium (supplemented DMEM) was added and the cells were grown for another 72 h. Cells were fixed with 25% trichloroacetic acid for 1 h at 4ºC and washed with water. After staining the cells with 0.4% sulforhodamine B in 1% (v/v) acetic acid for 15 min at room temperature, they were washed with 1% acetic acid and air-dried. The bound dye was solubilized with 10 mM unbuffered Tris and the absorbance was read at 492 nm. The absorbance was linear with cell concentrations from 1,000 to 200,000 cells/well. Separate wells were fixed 24 h after seeding to substract background staining. The anti-proliferative effects were determined and expressed as IC50 values which are the (pro)drug concentrations that gave 50% growth inhibition when compared to control cell growth.

Maximal tolerated dose (MTD)

The MTD (expressed in mg prodrug/kg mouse) of **DAU-GA3** and **DOX-GA3** was determined by intravenously injecting 1000, 500 or 250 mg/kg of prodrug in 3 groups of nude mice for each drug. The weight loss of the mice was followed in time. In was concluded that the MTD was given to the group of mice which received the highest dose of prodrug while showing not more than 5% weight loss.

Enzymatic prodrug activation rates

The enzymatic activation rates of the β-D-glucuronyl containing prodrugs were determined by incubation of 100 μM of prodrug in 0.1% BSA/PBS at pH = 6.8 with 0.03 U/mL human β-glucuronidase at 37ºC. The β-D-glucose and β-D-galactose based prodrugs were incubated with 0.3 U/mL bovine liver β-galactosidase[28] in 0.1% BSA/PBS at pH = 6.8 and 37ºC. Samples were prepared and analyzed on reversed phase SiO_2-C_{18} HPLC as described by us[14].

Pharmacokinetics

Protein binding properties of the halogenated prodrugs **25** (X = -Cl, -Br) were determined by the use of a Dianorm dialysis apparatus (Diachema, Zürich, Switzerland). Two 500 μL chambers were separated by a 5000 Da cut-off dialysis membrane. One chamber was filled with prodrug or drug diluted in 4% human serum albumin (HSA) in PBS at pH = 7.4. The second chamber was filled with PBS. After closing the chambers, the respective samples were incubated for 4 h at 37ºC under rotation. Samples from both chambers were diluted 20 times in 7.5% MeCN in PBS, the pH was adjusted to 3.5 with 1 M H_3PO_4. The fluorescence of the samples was recorded and compared to fluorescence properties of standard solutions of each (pro)drug in the same diluent (0.01 to 10 μM).

Circulation half-live were determined in BALB/c mice. 10 mg/kg of prodrug in 0.9% aqueous NaCl were intravenously injected into the animals. Over a period of 24 h, serial blood samples were collected from the eye plexus with the use of heparinized glass capillaries. Samples were centrifuged at 16,000 g for 4 min to prepare plasma. The samples were prepared and analyzed on reversed phase SiO_2-C_{18} HPLC as described[14] .

4.5.2 Chemistry

General

Daunorubicin-, doxorubicin- and idarubicin hydrochlorides were a generous gift of Pharmachemie BV (Haarlem, The Netherlands). Chromatotron model 7924-T Harrison Research (Palo Alto, California, USA) equipped with plates (thickness 2 mm, diameter 8.5 cm) made from Merck silicagel 60 PF_{254} which contains gypsum (art. 7749) was used when circular chromatography is indicated. When cation exchange material was indicated, amberlite resin IR-120 (Na) BDH (Poole, Dorset, England) was converted to the H^+-form using 1 N HCl prior to use. Reversed phase chromatography was performed with a liquid chromatography pump LC-410 (Kontron) using a pre-packed column (24 cm, diameter 11 mm) containing octadecylsilane (40-63 μm) Merck (Darmstadt, Germany). Prior to use the RP-C_{18} column was equilibrated with demineralized water. ^{1}H-NMR spectra at 400 MHz were obtained on a Bruker AM-400 spectrometer and 100 MHz spectra on a Bruker AM100 spectrometer. Chemical shifts are expressed in ppm downfield from internal standard Me_4Si. All solvents except MeCN and *i*-Pr_2O were dried before use: Et_3N, CCl_4, CH_2Cl_2 and PhMe were dried by distillation over CaH_2, pyridine by distillation over $CaCl_2$ and THF by distillation over $LiAlH_4$ or sodium. *i*-Pr_2NEt was dried over KOH pellets. In all cases demineralized H_2O was used. Allyl alcohol was dried by distillation over Mg/I_2.

General procedure #1: Modified Curtius reaction for conversion of carboxylic acids 18 (scheme 4.5) and 37 (scheme 4.7) to β-D-glycosyl carbamates.

500 mg of a carboxylic acid **18** or **37** was stirred overnight with 1.1 equiv. of $(PhO)_2P(O)N_3$ and 1.1 equiv. of Et_3N in 15 mL of dry PhMe under an argon atmosphere at room temperature. The reaction mixture was subsequently heated for 2 h until gas evolution was observed, occurring between 65-100ºC depending on the actual substrate. The reaction mixture was allowed to cool to ambient temperature and 0.5-0.75 equivalents [31] of the anomerically unprotected glycosyl donor **15** (scheme 4.4) or **42** (scheme 4.6) was added. The reaction mixture was stirred until **15** or **42** had almost disappeared on TLC (SiO_2,

Et_2O) and worked-up in the following fashion: The reaction mixture was diluted with 100 mL of Et_2O and successively washed with 200 mL portions of aqueous 0.5 N $KHSO_4$, with saturated aqueous $NaHCO_3$ (3x) and with brine. The organic layer was dried over Na_2SO_4 and evaporated. The residual material was purified by column chromatography (SiO_2, Et_2O/*n*-Hex 4/1) to yield 1-*O*-*β*-D-glycosyl carbamates **22** (scheme 4.5) or **39** (scheme 4.7) in good to excellent yield and 95-100 % *β*-diastereoselectivity.

General procedure #2: Removal of *tert.*-butyldimethylsilyl group from *β*-D-glycosyl carbamates 22 (scheme 4.5).

1.00 g of **22** was stirred in 100 mL of THF/H_2O/AcOH 1/1/1. The course of the deprotection reaction was followed on TLC (SiO_2, Et_2O). After *ca.* 3 h, the reaction mixture was diluted with 200 mL of H_2O and the THF was removed by evaporation. The aqueous layer was washed 5 times with 100 mL portions of CH_2Cl_2, the organic extracts were combined and washed successively with aqueous saturated $NaHCO_3$ until gas evolution ceased, and with brine. The organic layer was dried over Na_2SO_4 and evaporated. The resulting foam was sonicated in *i*-Pr_2O and collected by filtration. Traces of *t*-$BuMe_2SiOH$ were removed under reduced pressure to give **23** in almost quantitative yield.

General procedure #3: Coupling of pro-moieties 23 (scheme 4.5) and 35 (scheme 4.7) to anthracyclines.

100 mg of **23** or **35** was stirred with (1.1 equiv.) of di-*N*-*N'*-succinimidyl carbonate (**25**) and 2.5 equiv. of *i*-Pr_2NEt in 10 mL of CH_2Cl_2. Alternatively, **23** (Y = -$(CH_2)_5CH_3$) was coupled using 2.0 equiv. of *N*-succinimidyl chloroformate **26** and 3.0 equiv. of pyridine. After no starting material was detected on TLC (SiO_2, Et_2O) a solution of 1.1 equiv. of the anthracycline **DAU**-, **DOX**- or **IDA**-HCl and 2.5 equiv. of *i*-Pr_2NEt in 10 mL of DMF were added. The course of the reaction was monitored by TLC (SiO_2, CH_2Cl_2/EtOH 10/1). After all of the active ester starting material had disappeared, the reaction mixture was diluted with 200 mL of CH_2Cl_2 and washed successively with 100 mL portions of aqueous 0.5 N $KHSO_4$ (3x), with H_2O, with aqueous saturated $NaHCO_3$ (2x), H_2O (2x), and with brine. The organic layer was dried over Na_2SO_4 and evaporated. The resulting red residue was purified by circular chromatography using a chromatotron supplied with a 2 mm thick silica plate and mixtures of CH_2Cl_2/EtOH 10/1 (first run) and 30/1 (successive runs) until the protected prodrug **24** (scheme 4.5) or **40** (scheme 4.7) was a single spot on TLC (SiO_2, CH_2Cl_2/EtOH 20/1). After evaporation of the eluent, the resulting red product was sonicated in *i*-Pr_2O and collected by filtration to yield **24** or **40** as a red powder in low to good yield, depending on the starting material.

General procedure #4: Deprotection of the *β*-D-glycosyl specifier in the protected prodrugs 24 (scheme 4.5) and 40 (scheme 4.7).

To the protected prodrug **24** (scheme 4.5) or **40** (scheme 4.7) was added 6 equiv. of a 0.10 N LiOH solution in MeOH/H_2O/THF 2.5/1/0.5. The resulting deep blue solution was stirred at 0ºC under an argon atmosphere and progress of the deprotection reaction was continuously monitored by reversed phase TLC (SiO_2-C_{18}, MeCN/H_2O 1/1). After 15-90 min of deprotection, the reaction mixture was diluted to *ca.* twice the volume with H_2O, 25 vol% of THF was added and the mixture was neutralized by adding *ca.* 2 g of amberlite cation exchange material (H^+ form) per 100 mg of **24** or **40**. The amberlite material was removed by filtration and in case of a *β*-glucuronyl prodrug, *ca.* 10 equiv. of $NaHCO_3$ was added to convert the glucuronyl carboxylic acid group to the sodium salt. The MeOH and THF suspended in the water layer were removed by evaporation and the red aqueous product solution was transferred to a reversed phase column packed with RP-C_{18} material. To remove the excess of $NaCO_3$, the column was eluted with *ca.* 300 mL of H_2O. The column was washed with MeCN/H_2O 1/4 to elute the product and the MeCN was removed by evaporation. When the prodrug was not a single spot on TLC, the aqueous solution was transferred to the reversed phase column and purified again by eluting a MeCN/H_2O mixture in a ratio of 1/4 to 1/10. The MeCN was removed by evaporation and freeze drying of the aqueous product fraction afforded prodrugs **DAU-**, **DOX-** or **IDA-GA3**, **DAU-GsA3** or **DAU-GlA3** or **DAU-GA4** to **-GA9** or a small amount of impure **DAU-GA11** as red fluffy solids.

General procedure #5: α-Bromination of 33 (scheme 4.7).
300 mg of **33** was dissolved in 10 mL of CCl_4 and 1.1 equiv. of NBS and a catalytic amount of AIBN were added. The solution was irradiated for 1 h using a 250 W lamp. the temperature was allowed to raise to reflux. After cooling, the reaction mixture was filtered evaporated and dried under reduced pressure (0.1 mm Hg) to give the crude **34**. This was used in the next step without further purification.

General procedure #6: Hydrolysis of benzylic bromides 34 (scheme 4.7).
The crude **34** was dissolved in 10 mL of acetone and 10 mL (3 equiv.) of an aqueous 0.2 N $AgNO_3$ solution were added. Conversion of **34** to **35** was monitored on TLC (SiO_2, Et_2O), additional $AgNO_3$ was added when progress of the hydrolysis stopped. This procedure was repeated until no further reaction took place. The mixture was filtered and evaporated. The resulting oil was redissolved in CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$ (2x), with brine and dried over Na_2SO_4 and the CH_2Cl_2 was evaporated to give the crude **35**. This was used without further purification.

Synthesis of *N*-[4-(daunorubicin-*N*-carbonyl-oxymethyl)phenyl] *O*-β-D-glucuronyl carbamate sodium salt (DAU-GA3).
N-[4-(tert.-Butyldimethylsilyloxymethyl)phenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**22(GA3)**) [20] from **18** (Y = -H) [32] according to general procedure #1 as white needles from *i*-Pr_2O/*n*-Hex mp 128ºC in 95% yield and in 100% β-diastereoselectivity, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 0.00 (s, 6H, -$SiMe_2$-), 0.84 (s, 9H, -$SiCMe_3$), 1.95 (s, 9H, 3 -OAc), 3.64 (s, 3H, -CO_2Me), 4.12 (d, 1H, Gluc5-H, *J* = 9.3 Hz), 4.54 (s, 2H, ArCH_2-), 5.10-5.20 (m, 3H, Gluc2,3,4-H), 5.68 (d, 1H, Gluc1-H, *J* = 7.9 Hz), 6.63 (s, 1H, ArN*H*-), 7.15-7.30 (m, 4H, Ar2,3,5,6-H).

N-[4-(Hydroxymethyl)phenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**23(GA3)**) from **22(GA3)** according to general procedure #2 as a white powder, mp 173ºC in 92%. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.96 (s, 9H, 3 -OAc), 3.64 (s, 3H, -CO_2Me), 4.13 (d, 1H, Gluc5-H, *J* = 9.3 Hz), 4.61 (s, 2H, ArCH_2-), 5.00-5.35 (m, 3H, Gluc2,3,4-H), 5.71 (d, 1H, Gluc1-H, *J* = 7.5 Hz), 6.92 (s, 1H, ArN*H*-), 7.20-7.30 (m, 4H, Ar2,3,5,6H).

N-[4-(Daunorubicin-N-carbonyl-oxymethyl)phenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**24(DAU-GA3)**) from **23(GA3)** according to general procedure #3 in 84%, mp 163-164ºC. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.28 (d, 3H, 5'-Me, *J* = 6.6 Hz), 1.78 (dt, 1H, 2'$_{ax}$-H, *J* = 12.9 Hz *J* = 3.8 Hz), 1.87 (dd, 1H, 2'$_{eq}$-H, *J* = 13.3 Hz *J* = 5.8 Hz), 2.05 (s, 9H, 3 -OAc), 2.09 (dd, 1H, 8$_{ax}$-H, *J* = 15,2 Hz *J* = 3.7 Hz), 2.30 (d, 1H, 8$_{eq}$-H, *J* = 14.9 Hz), 2.41 (s, 3H, 9-C(O)Me), 2.88 (d, 1H, 10$_{ax}$-H, *J* = 18.8 Hz), 3.20 (d, 1H, 10$_{eq}$-H, *J* = 18.8 Hz), 3.67 (s, 1H, 4'-H), 3.72 (s, 3H, -CO_2Me), 3.88 (m, 1H, 3'-H), 4.05 (s, 3H, 4-OMe), 4.22 (d, 1H, Gluc5-H, *J* = 9.8 Hz), 4.15-4.25 (m, 1H, 5'-H), 4.48 (s, 1H, 9-OH), 4.89 (d, 1H, ArCH_aH$_b$-, *J* = 12.2 Hz), 4.95 (d, 1H, ArCH$_a$$H_b$-, *J* = 12.2 Hz), 5.15-5.30 (m, 4H, Gluc2,4-H 7-H 3'-NH-), 5.38 (t, 1H, Gluc3-H, *J* = 9.3 Hz), 5.47 (d, 1H, 1'-H, *J* = 3.1 Hz), 5.77 (d, 1H, Gluc1-H, *J* = 8.0 Hz), 7.16 (s, 1H, ArN*H*-), 7.18 (d, 2H, Ar3,5-H, *J* = 8.0 Hz), 7.24 (d, 2H, Ar2,6-H, *J* = 8.0 Hz) 7.37 (d, 1H, 3-H, *J* = 8.4 Hz), 7.76 (t, 1H, 2-H, *J* = 8.0 Hz), 8.00 (d, 1H, 1-H, *J* = 7.7 Hz), 13.22 (s, 1H, 11-OH), 13.94 (s, 1H, 6-OH).

N-[4-(Daunorubicin-N-carbonyl-oxymethyl)phenyl] O-β-D-glucuronyl carbamate sodium salt (**DAU-GA3**) from **24(DAU-GA3)** according to general procedure #4 in 81%, mp 175ºC (dec.). Anal.: calc. (found) for $C_{42}H_{43}N_2O_{20}Na\cdot 4\ H_2O$: C: 50.91 (50.73), H: 4.19 (4.96), N: 2.83 (2.94). MS (FAB$^+$) *m/z* = 942 ([M+1+Na]$^+$), 941 ([M+Na]$^+$), 920 ([M+1+H]$^+$), 919 ([M+H]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.11 (d, 3H, 5'-Me, *J* = 6.6 Hz), 1.47 (dd, 1H, 2'$_{eq}$-H, *J* = 12.1 Hz *J* = 3.4 Hz), 1.82 (dt, 1H, 2'$_{ax}$-H, *J* = 11.5 Hz *J* = 3.4 Hz), 2.07 (dd, 1H, 8$_{ax}$-H, *J* = 14.1 Hz *J* = 6.1 Hz), 2.19 (dd, 1H, 8$_{eq}$-H, *J* = 14.1 *J* = 3.1 Hz), 2.27 (s, 3H, -C(O)Me), 3.11 (d, 1H, 10$_{eq}$-H, *J* = 18.5 Hz), 3.13 (d, 1H, 10$_{ax}$-H, *J* = 18.5 Hz), 3.20-3.65 (m, 4H, 4'-H Gluc2,3,4-H), 3.71 (m, 1H, 3'-H), 3.95 (s, 3H, 4-OMe), 4.16 (q, 1H, 5'-H, *J* = 6.6 Hz), 4.70 (d, 1H, 4'-OH, *J* = 5.1 Hz), 4.75-5.35 (m, 4H, Gluc5-H Gluc2,3,4-OH), 4.84 (d, 1H, ArCH_aH$_b$-, *J* = 12.8 Hz), 4.88 (d, 1H, ArCH$_a$$H_b$-, *J* = 12.8 Hz), 4.90 (t, 1H, 7-H, *J* = 5.1 Hz), 5.20 (d, 1H, 1'-H, *J* = 3.0 Hz), 5.28 (d, 1H, Gluc1-H, *J* = 8.2 Hz), 5.52 (s, 1H, 9-OH), 6.84 (d, 1H, 3'-NH-, *J* = 8.0 Hz), 7.23 (d, 2H, Ar3,5-H, *J* = 8.4 Hz), 7.43 (d, 2H, Ar2,6-H, *J* = 8.4 Hz), 7.60 (dd, 1H, 3-H, *J* = 6.8 Hz *J* = 3.0 Hz), 7.80-7.90 (m, 2H, 1,2-H), 9.90 (s, 1H, ArN*H*-) 13.25 (s, 1H, 11-OH), 13.99 (s, 1H, 6-OH).

Synthesis of *N*-[4-(doxorubicin-*N*-carbonyl-oxymethyl)phenyl] *O*-β-D-glucuronyl carbamate sodium salt (DOX-GA3).

N-[4-(Doxorubicin-N-carbonyl-oxymethyl)phenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**24(DOX-GA3)**) from **23(GA3)** according to general procedure #3 in 69%, mp 165-167°C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.28 (d, 3H, 5'-Me, *J* = 6.5 Hz), 1.75-1.90 (m, 3H, 2'$_{eq}$-H 2'$_{ax}$-H 4'-OH), 2.05 (s, 9H, 3 -OAc), 2.14 (dd, 1H, 8$_{ax}$-H, *J* = 14.7 Hz *J* = 3.9 Hz), 2.32 (d, 1H, 8$_{eq}$-H, *J* = 14.7 Hz), 2.97 (d, 1H, 10$_{ax}$-H, *J* = 18.9 Hz), 3.06 (s, 1H, 14-OH), 3.24 (d, 1H, 10$_{eq}$-H, *J* = 18.9 Hz), 3.66 (m, 1H, 4'-H), 3.72 (s, 3H, -CO_2Me), 3.86 (m, 1H, 3'-H), 4.06 (s, 3H, 4-OMe), 4.13 (q, 1H, 5'-H, *J* = 6.5 Hz), 4.22 (d, 1H, Gluc5-H, *J* = 9.7 Hz), 4.55 (s, 1H, 9-OH), 4.75 (s, 2H, 9-C(O)CH_2-), 4.93 (d, 1H, ArCH_aH$_b$-, *J* = 12.4 Hz), 4.96 (d, 1H, ArCH$_a$$H_b$-, *J* = 12.4 Hz), 5.15-5.30 (m, 4H, 7-H 3'-NH- Gluc2,4-H), 5.36 (t, 1H, Gluc3-H, *J* = 9.3 Hz), 5.48 (d, 1H, 1'-H, *J* = 3.0 Hz), 5.77 (d, 1H, Gluc1-H, *J* = 8.0 Hz), 7.20 (d, 2H, Ar3,5-H, *J* = 7.0 Hz), 7.20-7.35 (m, 3H, Ar2,6-H ArN*H*-), 7.38 (d, 1H, 3-H, *J* = 8.5 Hz), 7.78 (t, 1H, 2-H, *J* = 8.0 Hz), 8.01 (d, 1H, 1-H, *J* = 7.8 Hz), 13.19 (s, 1H, 11-OH), 13.93 (s, 1H, 6-OH).

N-[4-(Doxorubicin-N-carbonyl-oxymethyl)phenyl] O-β-D-glucuronyl carbamate sodium salt (**DOX-GA3**) from **24(DOX-GA3)** according to general procedure #4 in 37%, mp 191°C (dec.). Anal.: calc. (found) for $C_{42}H_{43}N_2O_{21}Na\cdot 4\ H_2O$: C: 50.10 (50.31), H: 5.11 (4.81), N: 2.78 (3.00). MS (FAB$^+$) *m*/*z* = 958 ([M+1+Na]$^+$), 957 ([M+Na]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.12 (d, 3H, 5'-Me, *J* = 6.3 Hz), 1.47 (d, 1H, 2'$_{eq}$-H, *J* = 12.3 Hz), 1.83 (dt, 1H, 2'$_{ax}$-H, *J* = 12.9 Hz *J* = 3.5 Hz), 2.12 (dd, 1H, 8$_{ax}$-H, *J* = 14.2 Hz *J* = 5.6 Hz), 2.20 (d, 1H, 8$_{eq}$-H, *J* = 11.9 Hz), 2.95 (d, 1H, 10$_{eq}$-H, *J* = 18.8 Hz), 3.01 (d, 1H, 10$_{ax}$-H, *J* = 18.8 Hz), 3.05-3.60 (m, 4H, 4'-H Gluc2,3,4-H), 3.71 (m, 1H, 3'-H), 3.99 (s, 3H, 4-OMe), 4.15 (q, 1H, 5'-H, *J* = 6.3 Hz), 4.57 (s, 2H, 9-C(O)CH_2-), 4.62-4.71 (m, 1H, Gluc5-H), 4.68 (d, 1H, 4'-OH, *J* = 4.9 Hz), 4.80-5.30 (m, 4H, 14-OH Gluc2,3,4-OH), 4.88 (s, 2H, ArCH_2-), 4.94 (t, 1H, 7-H, *J* = 4.2 Hz), 5.21 (d, 1H, 1'-H, *J* = 2.7 Hz), 5.33 (d, 1H, Gluc1-H, *J* = 8.0 Hz), 5.46 (s, 1H, 9-OH), 6.83 (d, 1H, 3'-NH-, *J* = 8.0 Hz), 7.24 (d, 2H, Ar3,5-H, *J* = 8.1 Hz), 7.43 (d, 2H, Ar2,6-H, *J* = 8.1 Hz), 7.65 (t, 1H, 3-H, *J* = 4.8 Hz), 7.85-7.95 (m, 2H, 1,2-H), 9.91 (s, 1H, ArN*H*-) 13.28 (s, 1H, 11-OH), 14.03 (s, 1H, 6-OH).

Synthesis of *N*-[4-(idarubicin-*N*-carbonyl-oxymethyl)phenyl] *O*-β-D-glucuronyl carbamate sodium salt (IDA-GA3).

N-[4-(Idarubicin-N-carbonyl-oxymethyl)phenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**24(IDA-GA3)**) from **23(GA3)** according to general procedure #3 in 81%, mp 156-157 °C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.29 (d, 3H, 5'-Me, *J* = 6.6 Hz), 1.64 (s, 1H, 4'-OH), 1.80 (dt, 1H, 2'$_{ax}$-H, *J* = 13.1 Hz *J* = 3.9 Hz), 1.92 (dd, 1H, 2'$_{eq}$-H, *J* = 13.4 Hz *J* = 5.0 Hz), 2.04 (s, 9H, -OAc), 2.06 (dd, 1H, 8$_{ax}$-H, *J* = 15,2 Hz *J* = 3.8 Hz), 2.33 (d, 1H, 8$_{eq}$-H, *J* = 14.9 Hz), 2.42 (s, 3H, 9-C(O)Me), 2.97 (d, 1H, 10$_{ax}$-H, *J* = 18.8 Hz), 3.26 (d, 1H, 10$_{eq}$-H, *J* = 18.8 Hz), 3.68 (d, 1H, 4'-H, *J* = 6.3 Hz), 3.72 (s, 3H, -C(O)OMe), 3.91 (m, 1H, 3'-H), 4.22 (d, 1H, Gluc5-H, *J* = 9.6 Hz), 4.20-4.30 (m, 1H, 5'-H), 4.47 (s, 1H, 9-OH), 4.95 (s, 2H, ArCH_2-), 5.15-5.30 (m, 4H, 7-H 3'-NH- Gluc2,4-H), 5.36 (t, 1H, Gluc3-H, *J* = 9.3 Hz), 5.47 (d, 1H, 1'-H, *J* = 3.1 Hz), 5.77 (d, 1H, Gluc1-H, *J* = 8.0 Hz), 7.12 (s, 1H, ArN*H*-), 7.21 (d, 2H, Ar3,5-H, *J* = 7.8 Hz), 7.27 (d, 2H, Ar4,6-H, *J* = 7.8 Hz), 7.80-7.85 (m, 2H, 2,3-H), 8.30-8.35 (m, 2H, 1,4-H), 13.30 (s, 1 H, 11-OH), 13.56 (s, 1 H, 6-OH).

N-[4-(Idarubicin-N-carbonyl-oxymethyl)phenyl] O-β-D-glucuronyl carbamate sodium salt (**IDA-GA3**) from **24(IDA-GA3)** according to general procedure #4 in 79%, mp 187°C (dec.). Anal.: calc. (found) for $C_{41}H_{41}N_2O_{19}Na\cdot 3\ H_2O$: C: 52.23 (52.48), H: 5.02 (4.87), N: 2.97 (2.84). MS (FAB$^+$) *m*/*z* = 912 ([M+1+Na]$^+$), 911 ([M+Na]$^+$), 890 ([M+1+H]$^+$), 889 ([M+H]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.12 (d, 3H, 5'-Me, *J* = 6.3 Hz), 1.48 (d, 1H, 2'$_{eq}$-H, *J* = 12.6 Hz), 1.84 (dt, 1H, 2'$_{ax}$-H, *J* = 12.6 Hz *J* = 3.4 Hz), 2.12 (dd, 1H, 8$_{ax}$-H, *J* = 14.3 Hz *J* = 5.7 Hz), 2.22 (d, 1H, 8$_{eq}$-H, *J* = 14.3), 2.27 (s, 3H, 9-C(O)Me), 2.96 (d, 1H, 10$_{eq}$-H, *J* = 18.4 Hz), 3.03 (d, 1H, 10$_{ax}$-H, *J* = 18.4 Hz), 3.20-3.65 (m, 4H, 4'-H Gluc2,3,4-H), 3.73 (m, 1H, 3'-H), 4.18 (q, 1H, 5'-H, *J* = 6.3 Hz), 4.71 (d, 1H, 4'-OH, *J* = 5.5 Hz), 4.75-4. 85 (m, 1H, Gluc5-H), 4.87 (s, 2H, ArCH_2-), 4.95 (t, 1H, 7-H, *J* = 4.6 Hz), 5.22 (d, 1H, 1'-H, *J* = 2.6 Hz), 5.15-5.35 (m, 3H, Gluc2,3,4-OH), 5.35 (d, 1H, Gluc1-H, *J* = 8.1 Hz), 5.58 (s, 1H, 9-OH), 6.85 (d, 1H, 3'-NH-, *J* = 7.9 Hz), 7.24 (d, 2H, Ar3,5-H, *J* = 8.2 Hz), 7.43 (d, 2H, Ar2,6-H, *J* = 8.2 Hz), 7.95-8.05 (m, 2H, 2,3-H), 8.25- 8.35 (m, 2H, 1,4-H), 9.94 (s, 1H, ArN*H*-) 13.35 (s, 1H, 11-OH), 13.54 (s, 1H, 6-OH).

Synthesis of *N*-[4-(daunorubicin-*N*-carbonyl-oxymethyl)phenyl] *O*-β-D-glucosyl carbamate (DAU-GsA3).

N-[4-(tert.-Butyldimethylsilyloxymethyl)phenyl] O-β-(2,3,4,6-tetra-O-acetyl D-glucosyl) carbamate (**22(GsA3)**) from **18** (Y = -H) [32] according to general procedure #1 as white needles from *i*-Pr_2O/*n*-Hex mp 99-100ºC in 77% yield and in 100% β-diastereoselectivity. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 0.00 (s, 6H, -$SiMe_2$-), 0.84 (s, 9H, -$SiCMe_3$), 1.95 (s, 3H, -OAc), 1.96 (s, 3H, -OAc), 1.97 (s, 3H, -OAc), 2.00 (s, 3H, -OAc), 3.70-3.85 (m, 1H, Gluc5-H), 4.00 (dd, 1 H, Gluc6-$H_a$$H_b$-, *J* = 12.6 Hz, *J* = 2.1), 4.24 (dd, 1H, Gluc6-$H_a$$H_b$-, *J* = 12.6 Hz *J* = 4.3 Hz), 4.60 (s, 2H, ArCH_2-), 5.00-5.30 (m, 3H, Gluc2,3,4-H), 5.67 (d, 1 H, Gluc1-H, *J* = 7.8 Hz), 6.85 (s, 1 H, ArN*H*-), 7.17 (d, 2H, Ar3,5-H, *J* = 9.1 Hz), 7.28 (d, 2H, Ar2,6-H, *J* = 9.1 Hz).

N-[4-(Hydroxymethyl)phenyl] O-β-(2,3,4,6-tetra-O-acetyl D-glucosyl) carbamate (**23(GsA3)**) from **22(GsA3)** according to general procedure #2 in 68% as an oil, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.91, 1.92, 1.94 and 1.96 (4s, 12H, 4 -OAc), 3.70-3.85 (m, 1H, Gluc5-H), 3.98 (dd, 1H, Gluc6-$H_a$$H_b$-, *J* = 12.4 Hz, *J* = 2.0), 4.22 (dd, 1H, Gluc6-$H_a$$H_b$-, *J* = 12.4 Hz *J* = 4.3 Hz), 4.54 (s, 2H, ArCH_2-), 4.90-5.20 (m, 3H, Gluc2,3,4-H), 5.62 (d, 1H, Gluc1-H, *J* = 7.8 Hz), 6.93 (s, 1H, ArN*H*-), 7.15-7.25 (m, 4H, Ar2,3,5,6-H).

N-[4-(Daunorubicin-N-carbonyl-oxymethyl)phenyl] O-β-(2,3,4,6-tetra-O-acetyl D-glucosyl) carbamate (**24(DAU-GsA3)**) from **23(GsA3)** according to general procedure #3 in 58%, mp 145-148ºC. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.29 (d, 3H, 5'-Me, *J* = 6.5 Hz), 1.70-1.95 (m, 3H, 2'$_{eq}$-H 2'$_{ax}$-H 4'-OH), 2.02, 2.04, 2.06 and 2.07 (4s, 12H, 4 -OAc), 2.11 (dd, 1H, 8$_{ax}$-H, *J* = 14.8 Hz *J* = 3.9 Hz), 2.31 (d, 1H, 8$_{eq}$-H, *J* = 14.8 Hz), 2.41 (s, 3H, 9-C(O)Me), 2.91 (d, 1H, 10$_{ax}$-H, *J* = 18.7 Hz), 3.22 (d, 1H, 10$_{eq}$-H, *J* = 18.7 Hz), 3.65 (m, 1H, 4'-H), 3.85-3.95 (m, 2H, 3'-H Gluc5-H), 4.07 (s, 3H, 4-OMe), 4.12 (d, 1H, Gluc6-$H_a$$H_b$-, *J* = 11.7 Hz), 4.21 (q, 1H, 5'-H, *J* = 6.5 Hz), 4.31 (dd, 1H, Gluc6-$H_a$$H_b$-, *J* = 11.7 Hz *J* = 4.6 Hz), 4.48 (s, 1H, 9-OH), 4.92 (d, 1H, ArC$H_a$$H_b$-, *J* = 12.2 Hz), 4.97 (d, 1H, ArCH$_a$$H_b$-, *J* = 12.2 Hz), 5.10-5.35 (m, 5H, 7-H 3'-NH- Gluc2,3,4-H), 5.48 (d, 1H, 1'-H, *J* = 3.3 Hz), 5.75 (d, 1H, Gluc1-H, *J* = 8.1 Hz), 7.10 (s, 1H, ArN*H*-), 7.22 (d, 2H, Ar3,5-H, *J* = 7.9 Hz), 7.31 (d, 2H, Ar2,6-H, *J* = 7.9 Hz), 7.38 (d, 1H, 3-H, *J* = 8.6 Hz), 7.78 (t, 1H, 2-H, *J* = 8.0 Hz), 8.02 (d, 1H, 1-H, *J* = 7.5 Hz), 13.26 (s, 1H, 11-OH), 13.96 (s, 1H, 6-OH).

N-[4-(Daunorubicin-N-carbonyl-oxymethyl)phenyl] O-β-D-glucosyl carbamate (**DAU-GsA3**) from **24(DAU-GsA3)** according to general procedure #4 in 79%, mp 182-184ºC. Anal.: calc. (found) for $C_{42}H_{46}N_2O_{19}$·2.5 H_2O: C: 54.37 (54.45), H: 5.54 (5.24), N: 3.02 (2.88). MS (FAB$^+$) *m/z* = 906 ([M+1+Na]$^+$), 905 ([M+Na]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = -0.06 (s, 4H, Gluc2,3,4,6-OH), 1.13 (d, 3H, 5'-Me, *J* = 6.5 Hz), 1.47 (d, 1H, 2'$_{eq}$-H, *J* = 12.8 Hz), 1.83 (dt, 1H, 2'$_{ax}$-H, *J* = 12.8 Hz *J* = 3.5 Hz), 2.08 (dd, 1H, 8$_{ax}$-H, *J* = 14.4 Hz *J* = 5.4 Hz), 2.20 (d, 1H, 8$_{eq}$-H, *J* = 14.4 Hz), 2.27 (s, 3H, 9-C(O)Me),2.93 (s, 2H, Gluc6-H_2), 3.00-3.80 (m, 10H, 10$_{eq}$-H 10$_{ax}$-H 3',4'-H 9,4'-OH Gluc2,3,4,5-H), 3.97 (s, 3H, 4-OMe), 4.17 (q, 1H, 5'-H, *J* = 6.5 Hz), 4.87 (s, 2H, ArCH_2-), 4.92 (t, 1H, 7-H, *J* = 4.4 Hz), 5.21 (d, 1H, 1'-H, *J* = 2.6 Hz), 5.35 (d, 1H, Gluc1-H, *J* = 8.2 Hz), 6.83 (d, 1H, 3'-NH-, *J* = 8.0 Hz), 7.24 (d, 2H, Ar3,5-H, *J* = 8.3 Hz), 7.42 (d, 2H, Ar2,6-H, *J* = 8.3 Hz), 7.62 (dd, 1H, 3-H, *J* = 6.2 Hz *J* = 3.5 Hz), 7.85-7.90 (m, 2H, 1,2-H), 9.88 (s, 1H, ArN*H*-) 13.26 (s, 1H, 11-OH), 14.00 (s, 1H, 6-OH).

Synthesis of *N*-[4-(daunorubicin-*N*-carbonyl-oxymethyl)phenyl] *O*-β-D-galactosyl carbamate (DAU-GlA3).

N-[4-(tert.-Butyldimethylsilyloxymethyl)phenyl] O-β-(2,3,4,6-tetra-O-acetyl D-galactosyl) carbamate (**22(GlA3)**) from **18** (Y = -H) [32] according to general procedure #1 as a white foam mp 69-71ºC in 57% yield and in 100% β-diastereoselectivity, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 0.00 (s, 6H, -$SiMe_2$-), 0.84 (s, 9H, -$SiCMe_3$), 1.91 (s, 3H, -OAc), 1.96 (s, 3H, -OAc), 2.04 (s, 3H, -OAc), 2.08 (s, 3H, -OAc), 3.90-4.15 (m, 3H, Gal5,6-H_2), 4.61 (s, 2H, ArCH_2-), 5.03 (dd, 1H, Gal3-H, *J* = 3.2 Hz, *J* = 10.3 Hz), 5.25 (d, 1H. Gal2-H, *J* = 8.0 Hz), 5.36 (d, 1H, Gal4-H, *J* = 3.2 Hz), 5.66 (d, 1H, Gal1-H, *J* = 8.0 Hz), 6.93 (s, 1H, ArN*H*-).7.17 (d, 2H, Ar3,5-H, *J* = 8.8 Hz), 7.30 (d, 2H, Ar2,6-H, *J* = 8.8 Hz).

N-[4-(Hydroxymethyl)phenyl] O-β-(2,3,4,6-tetra-O-acetyl D-galactosyl) carbamate (**23(GlA3)**) from **22(GlA3)** according to general procedure #2 in 100% as an oil. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.92, 1.95, 1.99 and 2.04 (4s, 12H, 4 -OAc), 3.90-4.20 (m, 3H, Gal5,6-H), 4.55 (s, 2H, ArCH_2-), 5.05 (dd, 1H, Gal3-H, *J* = 10.1

Hz, *J* = 3.3 Hz), 5.27 (t, 1 H. Gal2-H, *J* = 7.9 Hz), 5.37 (d, 1H, Gal4-H, *J* = 2.9 Hz), 5.64 (d, 1H, Gal1-H, *J* = 7.8 Hz), 7.20 (d, 2H, Ar3,5-H, *J* = 10.1 Hz), 7.30 (d, 2H, Ar2,6-H, *J* = 10.1 Hz), 7.65 (s, 1H, ArN*H*-).

N-[4-(Daunorubicin-N-carbonyl-oxymethyl)phenyl] O-β-(2,3,4,6-tetra-O-acetyl D-galactosyl) carbamate (**24(DAU-GlA3)**) from **23(GlA3)** according to general procedure #3 in 45%, mp 170-173ºC. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.29 (d, 3H, 5'-Me, *J* = 6.5 Hz), 1.77 (s, 1H, 4'-OH), 1.78 (dt, 1H, $2'_{ax}$-H, *J* = 13.1 Hz *J* = 4.0 Hz), 1.88 (dd, 1H, $2'_{eq}$-H, *J* = 13.2 Hz *J* = 5.0 Hz), 2.00, 2.03, 2.05 and 2.17 (4s, 12H, 4 -OAc), 2.11 (dd, 1H, 8_{ax}-H, *J* = 14.8 Hz *J* = 4.0 Hz), 2.31 (d, 1H, 8_{eq}-H, *J* = 14.9 Hz), 2.42 (s, 3H, 9-C(O)Me), 2.91 (d, 1H, 10_{ax}-H, *J* = 18.8 Hz), 3.22 (d, 1H, 10_{eq}-H, *J* = 18.8 Hz), 3.67 (d, 1H, 4'-H, *J* = 5.9 Hz), 3.91 (bs, 1H, 3'-H), 4.07 (s, 3H, 4-OMe), 4.05-4.25 (m, 4H, 5'-H Gal5,6-H_2), 4.49 (s, 1H, 9-OH), 4.92 (d, 1H, ArCH_aH$_b$-, *J* = 12.3 Hz), 4.98 (d, 1H, ArCH$_a$$H_b$-, *J* = 12.3 Hz), 5.12 (dd, 1H, Gal3-H, *J* = 10.4 Hz *J* = 3.1 Hz), 5.19 (d, 1H, 3'-NH-, *J* = 8.3 Hz), 5.25 (s, 1H, 7-H), 5.35 (t, 1H, Gal2-H, *J* = 9.4 Hz), 5.44 (d, 1H, Gal4-H, *J* = 3.1 Hz), 5.49 (d, 1H, 1'-H, *J* = 3.4 Hz), 5.71 (d, 1H, Gal1-H, *J* = 8.3 Hz), 7.14 (s, 1H, ArN*H*-), 7.22 (d, 2H, Ar3,5-H, *J* = 7.9 Hz), 7.31 (d, 2H, Ar2,6-H, *J* = 7.9 Hz), 7.38 (d, 1H, 3-H, *J* = 8.5 Hz), 7.78 (t, 1H, 2-H, *J* = 8.1 Hz), 8.02 (d, 1H, 1-H, *J* = 7.7 Hz), 13.25 (s, 1H, 11-OH), 13.96 (s, 1H, 6-OH).

N-[4-(Daunorubicin-N-carbonyl-oxymethyl)phenyl] O-β-D-galactosyl carbamate (**DAU-GlA3**) from **24(DAU-GlA3)** according to general procedure #4 in 77%, mp 206-210ºC. Anal.: calc. (found) for $C_{42}H_{46}N_2O_{19}\cdot 2\,H_2O$: C: 54.90 (54.90), H: 5.48 (5.44), N: 3.05 (3.10). MS (FAB$^+$) *m/z* = 906 ([M+1+Na]$^+$), 905 ([M+Na]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.11 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.46 (dd, 1H, $2'_{eq}$-H, *J* = 12.8 Hz *J* = 3.8 Hz), 1.82 (dt, 1H, $2'_{ax}$-H, *J* = 12.8 Hz *J* = 3.5 Hz), 2.08 (dd, 1H, 8_{ax}-H, *J* = 14.1 Hz *J* = 5.7 Hz), 2.19 (d, 1H, 8_{eq}-H, *J* = 14.7 Hz), 2.25 (s, 3H, 9-C(O)Me), 2.93 (t, 2H, Gal6-H_2, *J* = 19.8 Hz), 3.30-3.75 (m, 9H, 10_{eq}-H 10_{ax}-H 3',4'-H 4'-OH Gal2,3,4,5-H), 3.97 (s, 3H, 4-OMe), 4.16 (q, 1H, 5'-H, *J* = 6.6 Hz), 4.50 (d, 1H, -OH, *J* = 3.9 Hz), 4.63 (d, 1H, -OH, *J* = 5.0 Hz), 5.68 (d, 1H, -OH, *J* = 5.5 Hz), 4.86 (s, 2H, ArCH_2-), 4.92 (t, 1H, 7-H, *J* = 4.3 Hz), 5.04 (d, 1H, -OH, *J* = 4.6 Hz), 5.20 (d, 1H, 1'-H, *J* = 2.6 Hz), 5.30 (d, 1H, Gal1-H, *J* = 8.0 Hz), 5.51 (s, 1H, 9-OH), 6.82 (d, 1H, 3'-NH-, *J* = 8.0 Hz), 7.23 (d, 2H, Ar3,5-H, *J* = 8.4 Hz), 7.421 (d, 2H, Ar2,6-H, *J* = 8.3 Hz), 7.62 (t, 1H, 3-H, *J* = 7.6 Hz), 7.85-7.90 (m, 2H, 1,2-H), 9.83 (s, 1H, ArN*H*-) 13.24 (s, 1H, 11-OH), 14.00 (s, 1H, 6-OH).

Synthesis of *N*-[2-(daunorubicin-*N*-carbonyl-oxymethyl)phenyl] *O*-β-D-glucuronyl carbamate sodium salt (DAU-GA4).

N-[2-(tert.-Butyldimethylsilyloxymethyl)phenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**22(GA4)**) from **27** (chart 4.4) according to general procedure #1 as a white foam mp 92-94ºC in 69% yield and in 100% β-diastereoselectivity, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 0.00 (s, 3H, SiMe_aMe$_b$-), 0.05 (s, 3H, SiMe$_a$$Me_b$-), 0.83 (s, 9H, $SiCMe_3$), 1.96 (s, 9H, 3 -OAc), 3.66 (s, 3H, -CO_2Me), 4.16 (d, 1H, Gluc5-H, *J* = 9.4 Hz), 4.60 (d, 1H, ArCH_aH$_b$-, *J* = 18.1 Hz), 4.72 (d, 1H, ArCH$_a$$H_b$-, *J* = 18.1 Hz), 5.00-5.35 (m, 3H, Gluc2,3,4-H), 5.76 (d, 1H, Gluc1-H, *J* = 7.8 Hz), 6.90-7.35 (m, 3H, Ar3,4,5-H), 7.90 (d, 1H, Ar6-H, *J* = 8.0 Hz), 8.61 (s, 1H, ArN*H*-).

N-[2-(Hydroxymethyl)phenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**23(GA4)**) from **22(GA4)** according to general procedure #2 in 68% as a white amorphous powder, mp 132ºC. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.96 (s, 9H, 3 -OAc), 3.64 (s, 3H, -CO_2Me), 4.11 (d, 1H, Gluc5-H, *J* = 9.4 Hz), 4.59 (s, 2H, ArCH_2-) , 5.10-5.20 (m, 3H, Gluc2,3,4-H), 5.74 (d, 1H, Gluc1-H, *J* = 7.5 Hz), 6.95-7.30 (m, 3H, Ar3,4,5-H), 7.70 (d, 1H, Ar6-H, *J* = 7.7 Hz), 8.15 (s, 1H, ArN*H*-).

N-[2-(Daunorubicin-N-carbonyl-oxymethyl)phenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**24(DAU-GA4)**) from **23(GA4)** according to general procedure #3 in 62%, mp 151-153ºC. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.30 (d, 3H, 5'-Me, *J* = 6.5 Hz), 1.80-1.85 (m, 3H, $2'_{eq}$-H $2'_{ax}$-H 4'-OH) 2.00, 2.04 and 2.05 (3s, 9H, 3 -OAc), 2.16 (dd, 1H, 8_{ax}-H, *J* = 14,8 Hz *J* = 4.1 Hz), 2.33 (d, 1H, 8_{eq}-H, *J* = 15.2 Hz), 2.43 (s, 3H, 9-C(O)Me), 2.95 (d, 1H, 10_{ax}-H, *J* = 18.9 Hz), 3.23 (d, 1H, 10_{eq}-H, *J* = 18.9 Hz), 3.67 (d, 1H, 4'-H, *J* = 6.7 Hz), 3.72 (s, 3H, -CO_2Me), 3.90 (m, 1H, 5'-H), 4.07 (s, 3H, 4-OMe), 4.20-4.25 (m, 2H, 3'-H Gluc5-H), 4.46 (s, 1H, 9-OH), 4.96 (d, 1H, ArCH_aH$_b$-, *J* = 12.6 Hz), 5.07 (d, 1H, ArCH$_a$$H_b$-, *J* = 12.6 Hz), 5.16 (t, 1H, Gluc2-H, *J* = 8.0 Hz), 5.25-5.35 (m, 4H, 7-H 3'-NH- Gluc3,4-H), 5.50 (m, 1H, 1'-H), 5.80 (d, 1H, Gluc1-H, *J* = 7.5), 7.08 (t, 1H, Ar4-H, *J* = 7.4 Hz), 7.26 (d, 1H, Ar3-H, *J* = 8.8 Hz), 7.32 (t, 1H, Ar5-H, *J* = 7.7 Hz), 7.39 (d, 1H, 3-H, *J* =

8.5 Hz), 7.78 (t, 1H, 2-H, *J* = 8.1 Hz), 7.84 (m, 1H, Ar6-H), 8.03 (d, 1H, 1-H, *J* = 7.7 Hz), 8.20 (s, 1H, ArN*H*-), 13.28 (s, 1H, 11-OH), 13.97 (s, 1H, 6-OH).

N-[2-(Daunorubicin-N-carbonyl-oxymethyl)phenyl] O-β-D-glucuronyl carbamate sodium salt (**DAU-GA4**) from **24(DAU-GA4)** according to general procedure #4 in 69%, mp 171ºC (dec.). Anal.: calc. (found) for $C_{42}H_{43}N_2O_{20}Na \cdot 3.5\ H_2O$: C: 51.38 (51.36), H: 5.13 (4.86), N: 2.85 (2.69). MS (FAB$^+$) *m/z* = 942 ([M+1+Na]$^+$), 941 ([M+Na]$^+$), 920 ([M+1+H]$^+$), 919 ([M+H]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.12 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.46 (dd, 1H, $2'_{eq}$-H, *J* = 12.6 Hz), 1.85 (dt, 1H, $2'_{ax}$-H, *J* = 12.6 Hz *J* = 3.5 Hz), 2.11 (dd, 1H, 8_{ax}-H, *J* = 14.4 Hz *J* = 5.8 Hz), 2.20 (d, 1H, 8_{eq}-H, *J* = 14.4 Hz), 2.26 (s, 3H, 9-C(O)Me), 2.93 (d, 1H, 10_{eq}-H, *J* = 18.2 Hz), 2.99 (d, 1H, 10_{ax}-H, *J* = 18.2 Hz), 3.15-3.65 (m, 4H, 4'-H Gluc2,3,4-H), 3.72 (m, 1H, 3'-H), 3.99 (s, 3H, 4-OMe), 4.17 (q, 1H, 5'-H, *J* = 6.4 Hz), 4.70-4.90 (m, 2H, 4'-OH, Gluc5-H), 4.94 (t, 1H, 7-H, *J* = 4.4 Hz), 4.98 (s, 2H, ArCH_2-), 5.20-5.25 (m, 2H, 2 Gluc-OH), 5.22 (d, 1H, 1'-H, *J* = 3.1 Hz), 5.32 (d, 1H, Gluc-OH, *J* = 5.5 Hz), 5.34 (d, 1H, Gluc1-H, *J* = 8.0 Hz), 5.55 (s, 1H, 9-OH), 7.03 (d, 1H, 3'-NH-, *J* = 8.0 Hz), 7.15 (t, 1H, Ar4-H, *J* = 7.3), 7.27 (t, 1H, Ar5-H, *J* = 7.7), 7.33 (d, 1H, Ar3-H, *J* = 7.6 Hz), 7.40 (d, 1H, Ar6-H, *J* = 7.8 Hz), 7.66 (dd, 1H, 3-H, *J* = 5.9 Hz *J* = 3.9 Hz), 7.90-7.95 (m, 2H, 1,2-H), 9.25 (s, 1H, ArN*H*-) 13.29 (s, 1H, 11-OH), 14.04 (s, 1H, 6-OH).

Synthesis of *N*-[4-(daunorubicin-*N*-carbonyl-1-oxyheptyl)phenyl] *O*-β-D-glucuronyl carbamate sodium salt (DAU-GA5).

4-(1-Hydroxyheptyl) bromobenzene (**29**, scheme 4.6). 0.78 g (0.9 equiv.) of Mg turnings, a crystal of I_2 and 30 mL of THF were brought into a 3-necked flask connected with a dropping funnel and a reflux condenser under an argon atmosphere. Approximately one third of 6.26 g (= 1 equiv.) of *n*-hexyl bromide in 10 mL of THF was added to the reaction vessel with the aid of a dropping funnel. The Grignard reagent was agitated and the rest of the *n*-hexyl bromide solution was added. When the magnesium had almost disappeared, the reaction mixture was cooled to -10ºC and 7.0 g (1.0 equiv.) of 4-bromobenzaldehyde **28** in 10 mL of THF were added slowly. Stirring was continued for 10 min at -10ºC and the reaction mixture was quenched on 20 g of ice containing 10 mL of 15% H_2SO_4 and the reaction mixture was extracted with 350 mL portions of Et_2O. The organic layer was washed with brine and dried over Na_2SO_4. Purification by column chromatography (SiO_2, *n*-Hex) yielded 2.9 g, 34%, of **22** as a colorless oil. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 0.86 (t, 3H, Alk7-H, *J* = 5.6 Hz), 1.10-1.70 (m, 10H, Alk2,3,4,5,6-H), 2.35 (bs, 1H, OH), 4.58 (t, 1H, Alk1-H, *J* = 6.3 Hz), 7.17 (d, 2H, Ar3,5-H, *J* = 8.3 Hz), 7.45 (d, 2H, Ar2,6-H, *J* = 8.3 Hz).

4-(1-tert.-Butyldimethylsilyloxyheptyl) bromobenzene (**30**). 655 mg of **29**, 247 mg (1.5 equiv.) of imidazole, 1.46 g (4.0 equiv.) of TBDMS-Cl and a catalytic amount of DMAP were dissolved in 20 mL of CH_2Cl_2 and stirred for 3 days under an argon atmosphere. After that, the reaction mixture was diluted with 200 mL of Et_2O, washed with 100 mL portions of aqueous 0.5 N $NaHSO_4$, aqueous saturated $NaHCO_3$ and brine. The organic layer was dried over $NaSO_4$ and evaporated. The product was purified by means of column chromatography (SiO_2, *n*-Hex) to give 602 mg of **30**, 65%, as a colorless oil. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = -0.14 (s, 3H, SiMe_aMe$_b$), 0.02 (s, 3H, SiMe$_a$$Me_b$), 0.67 (s, 9H, Si-*t*Bu), 0.70-1.75 (m, 13H, Alk2,3,4,5,6,7-H), 4.58 (t, 1H, Alk1-H, *J* = 5.8 Hz), 7.15 (d, 2H, Ar3,5-H, *J* = 8.5 Hz), 7.42 (d, 2H, Ar2,6-H, *J* = 8.4 Hz).

4-(1-tert.-Butyldimethylsilyloxyheptyl) benzoic acid (**18**, Y = -$(CH_2)_5CH_3$). To 1.1 mL (1.6 equiv.) of a 1.6 N *n*-BuLi solution and 1 mL of THF in a 3-necked flask connected with a dropping funnel under an argon atmosphere, 437 mg (= 1.0 equiv.) of **30** dissolved in 10 mL of THF were added slowly at -78ºC. The reaction mixture was stirred at -50ºC for 15 min and carefully added to a few grams of solid CO_2 in 10 mL of THF under an argon atmosphere. The reaction mixture was quenched with ice and 20 mL of aqueous 0.5 N $KHSO_4$ were added. The product was extracted with 100 mL portions of Et_2O (3x) and the combined organic extracts were washed with brine and dried over Na_2SO_4. The product was purified by means of column chromatography (SiO_2, CH_2Cl_2/EtOH 10/1) to yield 179 mg, 45%, of **18** (Y = -$(CH_2)_5CH_3$ as a colorless oil. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = -0.14 (s, 3H, SiMe_aMe$_b$), 0.03 (s, 3H, SiMe$_a$$Me_b$), 0.88 (s, 9H, Si-*t*-Bu), 0.70-1.70 (m, 13H, Alk2,3,4,5,6,7-H), 4.69 (t, 1H, Alk1-H, *J* = 5.5 Hz), 7.37 (d, 2H, Ar3,5-H, *J* = 8.1 Hz), 8.04 (d, 2H, Ar2,6-H, *J* = 8.1 Hz).

N-[4-(1-tert.-Butyldimethylsilyloxyheptyl)phenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**22(GA5)**) from **18** (Y = -$(CH_2)_5CH_3$) according to general procedure #1 as a colorless oil in 73%. Due to the racemic starting material, a diastereomeric mixture of **23(GA5)** must have been formed, conversely, this was not observed in the proton NMR spectrum. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = -0.23 (s, 3H, SiMe_aMe$_b$), 0.07 (s, 3H, SiMe$_a$$Me_b$), 0.59 (s, 9H, Si-*t*-Bu), 0.50-1.65 (m, 13H, Alk2,3,4,5,6,7-H), 1.98 (bs, 9H, 3 -OAc), 3.66 (s, 3H, -CO_2Me), 4.15 (d, 1H, Gluc5-H, *J* = 9.4 Hz), 4.51 (t, 1H, Alk1-H, *J* = 6.3 Hz), 5.10-5.45 (m, 3H, Gluc2,3,4-H), 5.73 (d, 1H, Gluc1-H, *J* = 7.6 Hz), 6.95 (bs, 1H, ArN*H*-), 7.13 (d, 2H, Ar3,5-H, *J* = 8.7 Hz), 7.03 (d, 2H, Ar2,6-H, *J* = 8.7).

N-[4-(1-Hydroxyheptyl)phenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**23(GA5)**) from **22(GA5)** according to general procedure #2 in 92%, as a colorless oil. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 0.80-1.90 (m, 13H, Alk2,3,4,5,6,7-H), 2.05 (s, 9H, 3 -OAc), 3.73 (s, 3H, -CO_2Me), 4.19 (d, 1H, Gluc5-H, *J* = 8.9 Hz), 4.63 (t, 1H, Alk1-H, *J* = 6.8 Hz), 5.15-5.30 (m, 3H, Gluc2,3,4-H), 5.76 (d, 1H, Gluc1-H, *J* = 7.4 Hz), 7.12 (bs, 1H, ArN*H*-), 7.22 (bs, 4H, Ar2,3,5,6-H).

N-[4-(Daunorubicin-N-carbonyl-(1-oxyheptyl))phenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**24(DAU-GA5)**) from **23(GA5)** according to general procedure #3 in 36%, mp 71°C. Because of chirality of the spacer -ArC*H(alkyl)(O)-, a diastereomeric mixture was formed in a ratio of approximately 1/1, determined by dividing the integrals of the clear singlets of the respective ArN*H*- signals. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 0.80-1.90 (m, 18H, Alk2,3,4,5,6,7-H 5'-Me 2'$_{ax}$-H 2'$_{eq}$-H), 2.00-2.05 (m, 9H, 3 -OAc), 2.09 (dd, 1H, 8$_{ax}$-H, *J* = 15,2 Hz *J* = 3.7 Hz), 2.30 (d, 1H, 8$_{eq}$-H, *J* = 14.9 Hz), 2.40 (bs, 3H, 9-C(O)Me), 2.88 (d, 1H, 10$_{ax}$-H, *J* = 18.8 Hz), 3.20 (d, 1H, 10$_{eq}$-H, *J* = 18.8 Hz), 3.65-3.90 (m, 5H, 3'-H 4'-H -CO_2Me), 4.06 and 4.09 (s, 3H, 4-OMe), 4.22 (bd, 1H,Gluc5-H, *J* = 9.8 Hz), 4.15-4.25 (m, 1H, 5'-H), 4.48 (s, 1H, 9-OH), 4.90-5.55 (m, 7H, ArC*H*alkyl- Gluc2,3,4-H 7-H 3'-NH- 1'-H), 5.79 (bd, 1H, Gluc1-H, *J* = 8.0 Hz), 6.77 and 6.86 (s, 1H, ArN*H*-), 7.15-7.25 (m, 4H, Ar2,3,5,6-H), 7.38 (bd, 1H, 3-H, *J* = 9.4 Hz), 7.77 (bt, 1H, 2-H, *J* = 9.0 Hz), 8.02 (bd, 1H, 1-H, *J* = 7.5 Hz), 13.26 and 13.28 (s, 1H, 11-OH), 13.95 and 13.98 (s, 1H, 6-OH).

N-[4-(Daunorubicin-N-carbonyl-(1-oxyheptyl))phenyl] O-β-D-glucuronyl carbamate sodium salt (**DAU-GA5**) from **24(DAU-GA5)** according to general procedure #4 in 39%, mp = 193°C (dec.). Anal.: calc. (found) for $C_{48}H_{55}N_2O_{20}Na\cdot 5\ H_2O$: C: 52.75 (52.73), H: 5.99 (5.69), N: 2.56 (2.70). MS (FAB$^+$) *m/z* = 1026 ([M+1+Na]$^+$), 1025 ([M+Na]$^+$), 1004 ([M+1+H]$^+$), 1003 ([M+H]$^+$). Because of chirality of the spacer -ArC*H(alkyl)(O)-, a diastereomeric mixture was formed in a ratio of approximately 1/0.2, determined by dividing the integrals of the clear singlets of the respective ArN*H*- signals in the proton-NMR spectrum. ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 0.76 and 0.81 (t, 3 H Alk7-H, *J* = 6.8 Hz resp. 6.7 Hz), 1.05-1.25 (m, 16H, 5'-Me Alk2,3,4,5,6,7-H), 1.39 and 1.46 (dd, 1H, 2'$_{eq}$-H, *J* = 12.3 Hz *J* = 4.1 Hz resp. 12.3 and 4.0 Hz), 1.79 (dt, 1H, 2'$_{ax}$-H, *J* = 12.9 Hz *J* = 3.9 Hz), 2.08 (m, 1H, 8$_{ax}$-H), 2.15 (d, 1H, 8$_{eq}$-H, *J* = 13.4 Hz), 2.23 (s, 3H, 9-C(O)Me), 2.90 (d, 1H, 10$_{eq}$-H, *J* = 18.4 Hz), 2.97 (d, 1H, 10$_{ax}$-H, J = 18.4 Hz), 3.00-3.45 (m, 4H, 4'-H Gluc2,3,4-H), 3.65 (m, 1H, 3'-H), 3.97 and 3.98 (s, 4-OMe), 4.12 (q, 1H, 5'-H, *J* = 6.2 Hz), 4.66 and 4.68 (d, 1H, 4'-OH, *J* = 5.4 Hz resp. *J* = 5.5 Hz), 4.91 (t, 1H, 7-H, *J* = 4.3 Hz), 5.00 (t, 1H, Alk1-H, *J* = 4.7 Hz), 5.18 and 4.21 (d, 1H, 1'-H, *J* = 1.6 Hz resp *J* = 2.4 Hz), 5.10-5.15 (m, 1H, Gluc5-H), 5.25 (d, 1H, Gluc1-H, *J* = 8.0 Hz), 5.50 (s, 1H, 9-OH), 6.72 (d, 1H, 3'-NH-, *J* = 7.8 Hz), 7.16 and 7.20 (d, 2H, Ar3,5-H, *J* = 8.5 Hz resp. *J* = 8.5 Hz), 7.37 and 7.41 (d, 2H, *J* = 8.2 Hz resp. *J* = 8.3 Hz), 7.63 (m, 1H, 3-H), 7.85-7.95 (m, 2H, 1,2-H), 9.78 and 9.84 (s, 1H, ArN*H*-), 13.19 (s, 1H, 11-OH), 14.01 (s, 1H, 6-OH).

Synthesis of *N*-methyl-[4-(daunorubicin-*N*-carbonyl-oxymethyl) 2-chlorophenyl] *O*-β-D-glucuronyl carbamate sodium salt (DAU-GA6).

N-(4-Methyl 2-chlorophenyl) O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**33**, X = -2-Cl). Since 4-methyl-2-chlorophenyl isocyanate (**32**, X = -2-Cl, scheme 4.7) was not commercially available, it was prepared from 500 mg of 4-methyl-2-chloroaniline-HCl (**31**, X = -2-Cl) by refluxing the latter compound for 2 h with 339 μL (1.0 equiv.) of diphosgene **41** in 20 mL of PhMe under an argon atmosphere. The reaction mixture was allowed to cool to ambient temperature and 1.5 mL (*ca.* 4 equiv.) of Et_3N were added to combine with the liberated HCl. 469 mg (0.5 equiv.) of **42** were added. After 0.5 h, **42** had disappeared and the reaction mixture was worked up according to general procedure #1, the product fraction was crystallized from *i*-Pr_2O to give 645 mg, 92%, of **33** (X = -2-Cl) as white crystals, mp 142-

143ºC. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 2.05 (s, 9H, 3 -OAc), 2.29 (s, 3H, Ar*Me*), 3.74 (s, 3H, -CO_2Me), 4.26 (d, 1H, Gluc5-H, *J* = 9.5 Hz), 5.10-5.55 (m, 3H, Gluc2,3,4-H), 5.86 (d, 1H, Gluc1-H, *J* = 7.4 Hz), 7.07 (d, 1H, Ar4-H, *J* = 8.3 Hz), 7.10-7.30 (m, 2H, Ar3-H, ArN*H*-), 7.92 (d, 1H, Ar6-H, *J* = 8.3 Hz).

N-[4-(Bromomethyl)-2-chlorophenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**34**, X = -2-Cl) from **33** (X = -2-Cl) according to general procedure #5**.** ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 2.05 (s, 9H, 3 -OAc), 3.75 (s, 3H, -CO_2Me), 4.25 (d, 1H, Gluc5-H, *J* = 9.2 Hz), 4.42 (s, 2H, ArCH_2-), 5.10-5.45 (m, 3H, Gluc2,3,4-H), 5.85 (d, 1H, Gluc1-H, *J* = 7.6 Hz), 7.10-7.50 (m, 3H, Ar3,5-H ArN*H*-), 8.09 (d, 1H, Ar6-H, *J* = 8.4 Hz).

N-[4-(Hydroxymethyl)-2-chlorophenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**35**, X = -2-Cl) from **34** (X = -2-Cl) according to general procedure #6 in 52%, from **33** (X = -2-Cl), ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.98 (s, 9H, 3 -OAc), 3.67 (s, 3H, -CO_2Me), 4.16 (d, 1H, Gluc5-H, *J* = 9.3 Hz), 4.56 (s, 2H, ArCH_2-), 5.00-5.40 (m, 3H, Gluc2,3,4-H), 5.75 (d, 1H, Gluc1-H, *J* = 7.5 Hz), 7.17 (d, 1H, Ar5-H, *J* = 8.4 Hz), 7.31 (bs, 2H, Ar3-H ArN*H*-), 7.37 (d, 1H, Ar6-H, *J* = 8.4 Hz).

N-[4-(Daunorubicin-N-carbonyl-oxymethyl)-2-chlorophenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**40**, X = -2-Cl) from **35** (X = -2-Cl) according to general procedure #3 in 28%, mp 150-155ºC. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.29 (d, 3H, 5'-Me, *J* = 6.6 Hz), 1.74 (dt, 1H, $2'_{ax}$-H, *J* = 13.1 Hz *J* = 4.1 Hz), 1.90 (dd, 1H, $2'_{eq}$-H, *J* = 13.1 Hz *J* = 4.8 Hz), 2.05 (s, 9H, 3 -OAc), 2.12 (dd, 1H, 8_{ax}-H, *J* = 14,9 Hz *J* = 4.1 Hz), 2.32 (d, 1H, 8_{eq}-H, *J* = 14.8 Hz), 2.41 (s, 3H, 9-C(O)Me), 2.95 (d, 1H, 10_{ax}-H, *J* = 18.8 Hz), 3.25 (d, 1H, 10_{eq}-H, *J* = 18.8 Hz), 3.65 (s, 1H, 4'-H), 3.73 (s, 3H, -CO_2Me), 3.87 (m, 1H, 3'-H), 4.08 (s, 3H, 4-OMe), 4.21 (d, 1H, Gluc5-H, *J* = 9.5 Hz), 4.20-4.25 (m, 1H, 5'-H), 4.44 (s, 1H, 9-OH), 4.95 (d, 1H, ArCH_2-), 5.10-5.30 (m, 4H, Gluc2,4-H 7-H 3'-NH-), 5.35 (t, 1H, Gluc3-H *J* = 8.9 Hz), 5.49 (d, 1H, 1'-H, *J* = 3.7 Hz), 5.81 (d, 1H, Gluc1-H, *J* = 7.9 Hz), 7.21 (d, 1H, Ar5-H, *J* = 8.3 Hz), 7.26 and 7.34 (2s, 2H, Ar3-H ArN*H*-), 7.40 (d, 1H, 3-H, *J* = 8.4 Hz), 7.79 (t, 1H, 2-H, *J* = 8.1 Hz), 8.00-8.10 (m, 2H, 1-H Ar6-H), 13.29 (s, 1H, 11-OH), 13.99 (s, 1H, 6-OH).

N-[4-(Daunorubicin-N-carbonyl-oxymethyl)-2-chlorophenyl] O-β-D-glucuronyl carbamate sodium salt (**DAU-GA6**) from **40** (X = -2-Cl) according to general procedure #4 in 37%, mp 175-179ºC. Anal.: calc. (found) for $C_{42}H_{42}N_2O_{20}ClNa{\cdot}5\ H_2O$: C: 48.38 (48.22), H: 5.02 (4.70), N: 2.69 (3.08). MS (FAB$^+$) *m/z* = 975 ([M+Na]$^+$), 955 ([M+2+H]$^+$), 954 ([M+1+H]$^+$), 953 ([M+H]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.12 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.48 (d, 1H, $2'_{eq}$-H, *J* = 12.6), 1.85 (t, 1H, $2'_{ax}$-H, *J* = 13.4 Hz), 2.05 (m, 2H, 8_{ax}-H 8_{eq}-H), 2.25 (s, 3H, 9-C(O)Me), 2.90 (m, 6H, 10_{eq}-H 10_{ax}-H 4'-H Gluc2,3,4-H), 3.73 (m, 1H, 3'-H), 3.98 (s, 3H, 4-OMe), 4.16 (m, 1H, 5'-H), 4.73 (d, 1H, 4'-OH, *J* = 5.1 Hz), 4.85 (m, 2H, ArCH_2-), 5.00 (d, 1H, 7-H, *J* = 5.2 Hz), 5.23 (bs, 1H, 1'-H), 5.27 (d, 1H, Gluc1-H, *J* = 7.9 Hz), 5.53 (s, 1H, 9-OH), 7.27 (d, 1H, Ar5-H, *J* = 7.3 Hz), 7.44 (s, 1H, Ar3-H), 7.57 (d, 1H, Ar6-H, *J* = 8.12 Hz), 7.64 (m, 1H, 3-H), 7.85-8.00 (m, 2H, 1,2-H), 9.22 (s, 1H, ArN*H*-).

Synthesis of *N*-methyl-[4-(daunorubicin-*N*-carbonyl-oxymethyl) 3-chlorophenyl] *O*-β-D-glucuronyl carbamate sodium salt (DAU-GA7).

N-(4-Methyl-3-chlorophenyl) O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**33**, X = -3-Cl)**.** 200 mg (1.19 mmol) of commercially available 4-methyl-3-chlorophenyl isocyanate **32** (X = -3-Cl) was dissolved in 10 mL of PhMe under an argon atmosphere. 199 mg (0.5 equiv.) of **42** and one drop of Et_3N was added. The course of the reaction was followed by means of TLC (SiO_2, Et_2O). After **42** had disappeared, the reaction mixture was taken to dryness (α/β = 1/10 by ^{1}H-NMR) and sonicated in Et_2O. The solid material was removed and the solution was concentrated and refrigerated overnight. 434 mg, 81%, of **33** (X = -3-Cl) was obtained as white crystals, mp 155ºC. The α-isomer stayed in solution. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.99 (s, 9H, 3 -OAc), 2.24 (s, 3H, Ar*Me*), 3.66 (s, 3H, -CO_2Me), 4.16 (d, 1H, Gluc5-H, *J* = 9.4 Hz), 5.00-5.45 (m, 3H, Gluc2,3,4-H), 5.71 (d, 1H, Gluc1-H, *J* = 7.6 Hz), 6.90-7.15 (m, 3H, Ar2,5,6-H), 7.39 (s, 1H, ArN*H*-).

N-[4-(Bromomethyl)-3-chlorophenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**34**, X = -3-Cl) from **33** (X = -3-Cl) according to general procedure #5. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.99 (s, 9H,

3 -OAc), 3.66 (s, 3H, -CO_2Me), 4.20 (d, 1H, Gluc5-H, *J* = 8.9 Hz), 4.47 (s, 2H, ArCH_2-), 5.05-5.45 (m, 3H, Gluc2,3,4-H), 5.71 (d, 1H, Gluc1-H, *J* = 7.5 Hz), 6.90-7.50 (m, 3H, Ar2,5,6-H), 7.63 (s, 1H, ArN*H*-).

N-[4-(Hydroxymethyl)-3-chlorophenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**35**, X = -3-Cl) from **34** (X = -3-Cl) according to general procedure #6 in 76% from **33** (X = -3-Cl), as a yellowish powder. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.99 (s, 9H, 3 -OAc), 3.66 (s, 3H, -CO_2Me), 4.16 (d, 1H, Gluc5-H, *J* = 9.3 Hz), 4.63 (s, 2H, ArCH_2-), 5.00-5.45 (m, 3H, Gluc2,3,4-H), 5.69 (d, 1H, Gluc1-H, *J* = 7.5 Hz), 7.10-745 (m, 3H, Ar2,4,5-H), 7.55 (s, 1H, ArN*H*-).

N-[4-(Daunorubicin-N-carbonyl-oxymethyl)-3-chlorophenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**40**, X = -3-Cl) from **35** (X = -3-Cl) according to general procedure #3 in 76%, mp 154-158ºC. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.29 (d, 3H, 5'-Me, *J* = 6.5 Hz), 1.78 (dt, 1H, 2'$_{ax}$-H, *J* = 13.0 Hz *J* = 3.9 Hz), 1.85-1.90 (m, 2H, 2'$_{eq}$-H 4'-OH), 2.05 (s, 9H, 3 -OAc), 2.05-2.15 (m, 1H, 8$_{ax}$-H), 2.28 (d, 1H, 8$_{eq}$-H, *J* = 14.9 Hz), 2.42 (s, 3H, 9-C(O)Me), 2.87 (d, 1H, 10$_{ax}$-H, *J* = 19.0 Hz), 3.19 (d, 1H, 10$_{eq}$-H, *J* = 18.8 Hz), 3.68 (bs, 1H, 4'-H), 3.74 (s, 3H, -CO_2Me), 3.87 (bs, 1H, 3'-H), 4.05 (s, 3H, 4-OMe), 4.15-4.20 (m, 1H, 5'-H), 4.24 (d, 1H,Gluc5-H, *J* = 9.8 Hz), 4.46 (s, 1H, 9-OH), 4.97 (d, 1H, ArCH_aH$_b$-, *J* = 12.7 Hz), 5.01 (d, 1H, ArCH$_a$$H_b$-, *J* = 12.7 Hz), 5.12-5.40 (m, 4H, Gluc3,4-H 7-H 3'-NH-), 5.48 (d, 1H, 1'-H, *J* = 2.4 Hz), 5.73 (d, 1H, Gluc1-H, *J* = 8.0 Hz), 7.00-7.50 (m, 5H, 3-H Ar2,5,6-H ArN*H*-), 7.76 (t, 1H, 2-H, *J* = 8.1 Hz), 8.00 (d, 1H, 1-H, *J* = 8.2 Hz), 13.24 (s, 1H, 11-OH), 13.95 (s, 1H, 6-OH).

N-[4-(Daunorubicin-N-carbonyl-oxymethyl)-3-chlorophenyl] O-β-D-glucuronyl carbamate sodium salt (**DAU-GA7**) from **40** (X = -3-Cl) according to general procedure #4 in 67%, mp 205ºC (dec.). Anal.: calc. (found) for $C_{42}H_{42}N_2O_{20}ClNa\cdot 6\ H_2O$: C: 47.53 (47.25), H: 5.13 (4.75), N: 2.64 (2.64). MS (FAB$^+$) *m*/*z* = 977 ([M+2+Na]$^+$), 976 ([M+1+Na]$^+$), 975 ([M+Na]$^+$), 955 ([M+2+H]$^+$), 954 ([M+1+H]$^+$), 953 ([M+H]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.11 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.46 (dd, 1H, 2'$_{eq}$-H, *J* = 12.0 Hz *J* = 3.6 Hz), 1.81 (dd, 1H, 2'$_{ax}$-H, *J* = 12.7 Hz *J* = 3.2 Hz), 2.08 (dd, 1H, 8$_{ax}$-H, *J* = 13.9 Hz *J* = 5.2 Hz), 2.17 (d, 1H, 8$_{eq}$-H, *J* = 13.6 Hz), 2.24 (s, 3H, 9-C(O)Me), 3.00 (m, 6H, 10$_{eq}$-H 10$_{ax}$-H 4'-H Gluc2,3,4-H), 3.70 (m, 1H, 3'-H), 3.96 (s, 3H, 4-OMe), 4.13 (q, 1H, 5'-H, *J* = 6.5 Hz), 4.69 (d, 1H, 4'-OH, *J* = 3.9 Hz), 4.94 (s, 2H, ArCH_2-), 5.07 (bs, 1H, 7-H), 5.21 (s, 1H, 1'-H), 5.28 (d, 1H, Gluc1-H, *J* = 8.1 Hz), 5.51 (s, 1H, 9-OH), 6.95 (d, 1H, 3'-NH-, *J* = 7.9 Hz), 7.23 (d, 1H, Ar5-H, *J* = 8.4 Hz), 7.35-7.40 (m, 2H, Ar2,6-H), 7.55-7.60 (m, 2H, 3-H Ar2-H), 7.80-7.95 (m, 2H, 1,2-H), 10.14 (s, 1H, ArN*H*-).

Synthesis of *N*-methyl-[4-(daunorubicin-*N*-carbonyl-oxymethyl)-2-bromophenyl] *O*-β-D-glucuronyl carbamate sodium salt (DAU-GA8).

N-[4-(Methyl)-2-bromophenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**33**, X = -2-Br). 263 mg (1.20 mmol) of **31** (X = -2-Br) and 145 μL (1.0 equiv.) of diphosgene **41** were dissolved in 10 mL of PhMe and the reaction mixture was refluxed for 2 h under an argon atmosphere. When HCl evolution ceased, the mixture was cooled to 0ºC and 834 μL (5.0 equiv.) of Et_3N and 200 mg (0.5 equiv.) of **42** were added. The reaction mixture was stirred for 1 h at 0ºC and processed according to general procedure #1. The crude product was purified by means of column chromatography (SiO_2, Et_2O/*n*-Hex 1/1) to give 310 mg (95% from **42**) of **33** (X = -2-Br), mp 139ºC. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 2.05 (s, 9H, 3 -OAc), 2.30 (s, 3H, Ar*Me*), 3.75 (s, 3H, -CO_2Me), 4.22 (d, 1H, Gluc5-H, *J* = 9.1 Hz), 5.10-5.45 (m, 3H, Gluc2,3,4-H), 5.83 (d, 1H, Gluc1-H, *J* = 7.8 Hz), 7.15-7.45 (m, 2H, Ar5-H ArN*H*-), 7.35 (d, 1H, Ar3-H, *J* = 2.0 Hz) 7.93 (d, Ar6-H, *J* = 8.3 Hz).

N-[4-(Bromomethyl)-2-bromophenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**34**, X = -2-Br) from **33** (X = -2-Br) according to general procedure #5, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 2.05 (s, 9H, 3 -OAc), 3.75 (s, 3H, -CO_2Me), 4.23 (d, 1H, Gluc5-H, *J* = 9.1 Hz), 4.42 (s, 3H, ArCH_2-), 5.10-5.45 (m, 3H, Gluc2,3,4-H), 5.83 (d, 1H, Gluc1-H, *J* = 7.8 Hz), 7.35 (dd, 1H, Ar5-H, *J* = 8.4 Hz *J* = 2.0 Hz), 7.40 (s, 1H, ArN*H*-), 7.59 (d, 1H, Ar3-H, *J* = 2.0 Hz), 8.09 (d, Ar6-H, *J* = 8.4 Hz).

N-[4-(Hydroxymethyl)-2-bromophenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**35**, X = -2-Br) from **34** (X = -2-Br) according to general procedure #6 in 80% from **33** (X = -2-Br), as an oil, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 2.04 (s, 9H, 3 -OAc), 3.75 (s, 3H, -CO_2Me), 4.22 (d, 1H, Gluc5-H, *J* = 9.2 Hz), 4.65

(s, 3H, ArCH_2-), 5.20-5.40 (m, 3H, Gluc2,3,4-H), 5.83 (d, 1H, Gluc1-H, *J* = 7.5 Hz), 7.20-7.40 (m, 2H, Ar5-H ArN*H*-), 7.57 (d, 1H, Ar3-H, *J* = 1.8 Hz), 8.07 (d, Ar6-H, *J* = 8.1 Hz).

N-[4-(Daunorubicin-N-carbonyl-oxymethyl)-2-bromophenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**40**, X = -2-Br) from **35** (X = -2-Br) according to general procedure #3 in 14%, mp 149ºC. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.22 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.58 (s, 1H, 4'-OH), 1.70 (dt, 1H, 2'$_{eq}$-H, *J* = 13.2 Hz *J* = 4.2 Hz), 1.82 (dd, 2'$_{ax}$, 1H, *J* = 13.3 Hz *J* = 5.1 Hz), 1.95 (s, 9H, 3 -OAc), 2.04 (dd, 1H, 8$_{ax}$-H, *J* = 14.9 Hz *J* = 4.1 Hz), 2.24 (d, 1H, 8$_{eq}$-H, *J* = 14.4 Hz), 2.34 (s, 3H, 9-C(O)Me), 2.83 (d, 1H, 10$_{ax}$-H, *J* = 18.8 Hz), 3.16 (d, 1H, 10$_{eq}$-H, *J* = 18.8 Hz), 3.59 (m, 1H, 4'-H), 3.68 (s, 3H, -CO_2Me), 3.81 (m, 1H, 3'-H), 4.01 (s, 3H, 4-OMe), 4.15 (d, 1H, Gluc5-H, *J* = 9.6 Hz), 4.10-4.20 (m, 1H, 5'-H), 4.38 (s, 1H, 9-OH), 4.86 (d, 1H, ArCH_aH$_b$-, *J* = 12.5 Hz), 4.89 (d, 1H, ArCH$_a$$H_b$-, *J* = 12.5 Hz), 5.05-5.25 (m, 4H, Gluc2,4-H 7-H 3'-NH-), 5.28 (t, 1H, Gluc3-H, *J* = 9.3 Hz), 5.42 (d, 1H, 1'-H, *J* = 3.7 Hz), 5.74 (d, 1H, Gluc1-H, *J* = 8.0 Hz), 7.15-7.25 (m, 2H, Ar5,6-H), 7.32 (d, 1H, 3-H, *J* = 8.6 Hz), 7.41 (s, 1H, Ar3-H), 7.71 (t, 1H, 2-H, *J* = 8.0 Hz), 7.96 (d, 1H, 1-H, *J* = 7.6 Hz), 7.90-8.00 (m, 1H, ArN*H*-), 13.19 (s, 1H, 11-OH), 13.90 (s, 1H, 6-OH).

N-[4-(Daunorubicin-N-carbonyl-oxymethyl)-2-bromophenyl] O-β-D-glucuronyl carbamate sodium salt (**DAU-GA8**) from **40** (X = -2-Br) according to general procedure #4 in 34%, mp 213ºC (dec.). Anal.: calc. (found) for $C_{42}H_{42}N_2O_{20}BrNa\cdot 3\ H_2O$: C: 47.96 (47.75), H: 4.60 (4.59), N: 2.66 (3.08). MS (FAB$^+$) *m*/*z* = 1021 ([M+2+Na]$^+$), 1019 ([M+Na]$^+$), 999 ([M+2+H]$^+$), 997 ([M+H]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.13 (d, 3H, 5'-Me, *J* = 6.5 Hz), 1.48 (dd, 1H, 2'$_{eq}$-H, *J* = 12.4 Hz *J* = 5.3 Hz), 1.83 (dt, 1H, 2'$_{ax}$-H, *J* = 13.3 Hz *J* = 3.5 Hz), 2.10-2.20 (m, 2H, 8$_{ax}$-H 8$_{eq}$-H), 2.26 (s, 3H, 9-C(O)Me), 2.94 (d, 1H, 10$_{ax}$-H, *J* = 18.5 Hz), 3.10-3.65 (m, 5H, 10$_{eq}$-H 4'-H Gluc2,3,4-H), 3.73 (m, 1H, 3'-H), 3.99 (s, 3H, 4-OMe), 4.18 (q, 1H, 5'-H, *J* = 6.3 Hz), 4.72 (d, 1H, 4'-OH, *J* = 5.6 Hz), 4.75-5.20 (m, 2H, 7-H Gluc5-H), 4.93 (s, 2H, ArCH_2-), 5.22 (d, 1H, 1'-H, *J* = 3.5 Hz), 5.27 (d, 1H, Gluc1-H, *J* = 8.1 Hz), 5.55 (s, 1H, 9-OH), 6.98 (d, 1H, 3'-NH-, *J* = 8.0 Hz), 7.31 and 7.52 (2d, 2H, Ar5-H or Ar6-H, *J* = 8.4 resp. 8.2 Hz), 7.61 (s, 1H, Ar3-H), 7.67 (t, 1H, 3-H, *J* = 7.8 Hz), 7.90-8.00 (m, 2H, 1,2-H). 8.31 (s, 1H, ArN*H*-).

Synthesis of *N*-methyl-[4-(daunorubicin-*N*-carbonyl-oxymethyl)-3-bromophenyl] *O*-β-D-glucuronyl carbamate sodium salt (DAU-GA9).

3-Bromoterephthalic acid allyl ester (**37**, X = -3-Br). 3.0 g (12 mmol) of bromo terephthalic acid (**36**, X = -Br) were dissolved in 150 mL of THF under an argon atmosphere and 1.25 mL (1.5 equiv.) of AllOH and a catalytic amount of DMAP was added. After cooling the mixture to 0ºC, 2.2 g (1.0 equiv.) of DCC were added and the reaction mixture was stirred at 0ºC for 2 h and overnight at room temperature. The reaction mixture was filtrated and evaporated, the product was purified by means of column chromatography (SiO_2, CH_2Cl_2/EtOH 10/1) and crystallized from *n*-Hex to yield 893 mg, 26%, of **37** (X = -3-Br) as white needles, mp 117ºC. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 4.88 (d, 2H, All1-H, *J* = 5.6 Hz), 5.30-5.55 (m, 2H, 2 All3-H), 5.90-6.25 (m, 1H, All2-H), 7.79 (d, 1H, Ar5-H, *J* = 8.0 Hz), 8.10 (dd, 1H, Ar6-H, *J* = 8.0 Hz *J* = 1.5 Hz), 8.40 (d, 1H, Ar2-H, *J* = 1.5 Hz).

N-[4-(Carboxylic acid allyl ester)-3-bromophenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**39** (X = -3-Br) from **37** (X = -3-Br) according to general procedure #1 in 80% as a white powder, mp 61ºC. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 2.05 (s, 9H, 3 -OAc), 3.72 (s, 3H, -CO_2Me), 4.24 (d, 1H, Gluc5-H, *J* = 9.5 Hz), 4.79 (d, 2H, 2 All1-H, *J* = 5.6 Hz), 5.10-5.50 (m, 5H, Gluc2,3,4-H All3-H), 5.77 (d, 1H, Gluc1-H, *J* = 7.6 Hz), 5.70-6.25 (m, 1H, All2-H), 7.34 (dd, 1H, Ar6-H, *J* = 8.6 Hz *J* = 2.2 Hz), 7.58 (s, 1H, ArN*H*-), 7.74 (d, 1H, Ar2-H, *J* = 2.2 Hz), 7.83 (d, Ar5-H, *J* = 8.6 Hz).

N-[4-(Hydroxymethyl)-3-bromophenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**35**, X = -3-Br). 263 mg (0.427 mmol) of **39** (X = -3-Br), 186 μL (5 equiv.) of morpholine in 20 mL of THF were stirred for 2 h at room temperature while bubbling argon through the solution. After that, a catalytic amount of $Pd(PPh_3)_4$ was added. After 15 min **39** (X = -3-Br) had disappeared on TLC (SiO_2, Et_2O) and the reaction mixture was diluted with 100 mL of Et_2O and washed with 100 mL portions of aqueous 0.5 N $KHSO_4$ (3x) and with brine. The organic layer was dried over Na_2SO_4 and evaporated to obtain 246 mg of the carboxylic acid in a quantitative yield, mp 110ºC. This was used without further purification in the next reduction reaction. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 2.04 (s, 9H, 3 -OAc), 3.73 (s, 3H, -CO_2Me), 4.23

(d, 1H, Gluc5-H, *J* = 9.5 Hz), 5.10-5.40 (m, 3H, Gluc2,3,4-H), 5.77 (d, 1H, Gluc1-H, *J* = 7.6 Hz), 7.36 (dd, 1H, Ar6-H, *J* = 8.6 Hz *J* = 2.2 Hz), 7.56 (s, 1H, ArN*H*-), 7.79 (d, 1H, Ar2-H, *J* = 2.2 Hz) 7.96 (d, Ar5-H, *J* = 8.6 Hz).
153 mg (0.265 mmol) of the latter carboxylic acid were stirred in 15 mL of THF at room temperature under an argon atmosphere. 1.33 mL (5.0 equiv.) of a 1 M BH_3-THF solution were added slowly over a period of 20 min. The reaction mixture was stirred overnight at room temperature. When the acid had almost disappeared on TLC (SiO_2, Et_2O), the reaction mixture was quenched with 15 mL of H_2O. When gas evolution ceased, the reaction mixture was diluted with 200 mL of EtOAc and washed with 100 mL portions of saturated aqueous $NaHCO_3$ (3x) and with brine. The organic layer was dried over Na_2SO_4 and evaporated to yield 140 mg, 94%, of the pure **35** (X = -3-Br) as an oil, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 2.00 (s, 9H, 3 -OAc), 3.67 (s, 3H, -CO_2Me), 4.14 (d, 1H, Gluc5-H, *J* = 9.6 Hz), 4.63 (s, 2H, ArCH_2-), 5.00-5.30 (m, 3H, Gluc2,3,4-H), 5.69 (d, 1H, Gluc1-H, *J* = 7.6 Hz), 7.15-7.40 (m, 3H, Ar5,6-H ArN*H*-), 7.63 (d, 1H, Ar2-H, *J* = 2.2 Hz).

N-[4-(Daunorubicin-N-carbonyl-oxymethyl)-3-bromophenyl] O-β-(methyl 2,3,4-tri-O-acetyl D-glucuronyl) carbamate (**40**, X = -3-Br) from **35** (X = -3-Br) according to general procedure #3 in 56%, mp 147°C. ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.13 (d, 3H, 5'-Me, *J* = 6.5 Hz), 1.62 (s, 1H, 4'-OH), 1.81 (dt, 1H, 2'$_{eq}$-H, *J* = 13.2 Hz *J* = 3.3 Hz), 1.89 (dd, 1H, 2'$_{ax}$-H, *J* = 13.2 Hz *J* = 5.0 Hz), 2.06 (s, 9H, 3 -OAc), 2.09 (dd, 1H, 8$_{ax}$-H, *J* = 14.7 Hz *J* = 3.8 Hz), 2.30 (d, 1H, 8$_{eq}$-H, *J* = 14.7 Hz), 2.42 (s, 3H, C(O)Me), 2.88 (d, 1H, 10$_{ax}$-H, *J* = 18.8 Hz), 3.20 (d, 1H, 10$_{eq}$-H, *J* = 18.7 Hz), 3.69 (m, 1H, 4'-H), 3.72 (s, 3H, -CO_2Me), 3.90 (m, 1H, 3'-H), 4.04 (s, 3H, 4-OMe), 4.20-4.25 (m, 2H, 5'-H Gluc5-H), 4.50 (s, 1H, 9-OH), 4.95 (d, 1H, ArCH_aH$_b$-, *J* = 12.8 Hz), 5.00 (d, 1H, ArCH$_a$$H_b$-, *J* = 12.8 Hz), 5.15-5.30 (m, 4H, Gluc2,4-H 7-H 3'-NH-), 5.40 (t, 1H, Gluc3-H, *J* = 9.4 Hz), 5.47 (d, 1H, 1'-H, *J* = 2.7 Hz), 5.75 (d, 1H, Gluc1-H, *J* = 8.0 Hz), 7.08 and 7.16 (2d, 2H, Ar5-H or Ar6-H, *J* = 8.2 Hz), 7.36 (d, 1H, 3-H, *J* = 8.5 Hz), 7.54 or 7.60 (2s, 2H, ArN*H*- Ar2-H), 7.76 (t, 1H, 2-H, *J* = 8.0 Hz), 7.99 (d, 1H, 1-H, *J* = 7.8 Hz), 13.22 (s, 1H, 11-OH), 13.94 (s, 1H, 6-OH).

N-[4-(Daunorubicin-N-carbonyl-oxymethyl)-3-bromophenyl] O-β-D-glucuronyl carbamate sodium salt (**DAUGA9**) from **40** (X = -3-Br) according to general procedure #4 in 50%, mp 181°C. Anal.: calc. (found) for $C_{42}H_{42}N_2O_{20}BrNa\cdot 5\ H_2O$: C: 46.38 (46.37), H: 4.82 (4.49), N: 2.58 (2.68). MS (FAB$^+$) *m/z* = 1021 ([M+2+Na]$^+$), 1019 ([M+Na]$^+$), 999 ([M+2+H]$^+$), 997 ([M+H]$^+$). ^{1}H-NMR (400 MHz, $(CD_3)_2SO$) δ (ppm) = 1.11 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.47 (dd, 1H, 2'$_{eq}$-H, *J* = 11.6 Hz *J* = 3.1 Hz), 1.82 (dt, 1H, 2'$_{ax}$-H, *J* = 12.4 Hz *J* = 3.3 Hz), 2.08 (dd, 1H, 8$_{ax}$-H, *J* = 13.4 Hz *J* = 5.0 Hz), 2.19 (dd, 1H, 8$_{eq}$-H, *J* = 13.4 Hz *J* = 2.5 Hz), 2.25 (s, 3H, 9-C(O)Me), 3.05-3.65 (m, 7H, 10$_{ax}$-H 10$_{eq}$-H 3',4'-H Gluc2,3,4-H), 3.97 (s, 3H, 4-OMe), 4.16 (q, 1H, 5'-H, *J* = 6.6 Hz), 4.70 (d, 1H, 4'-OH, *J* = 5.5 Hz), 4.85 -4.95 (m, 1H, Gluc5-H), 4.90 (d, 1H, ArCH_aH$_b$-, *J* = 13.5 Hz), 4.93 (d, 1H, ArCH$_a$$H_b$-, *J* = 13.5 Hz), 5.20-5.25 (m, 2H, 1'-H 7-H), 5.28 (d, 1H, Gluc1-H, *J* = 8.0 Hz), 5.54 (s, 1H, 9-OH), 6.96 (d, 1H, 3'-NH-, *J* = 7.9 Hz), 7.36 and 7.44 (2d, 2H, Ar5-H or Ar6-H, *J* = 8.4 Hz), 7.63 (t, 1H, 3-H, *J* = 4.5 Hz), 7.77 (s, 1H, Ar2-H), 7.85 -7.90 (m, 2H, 1,2-H), 10.15 (s, 1H, ArN*H*-), 13.23 (s, 1H, 11-OH), 14.01 (s, 1H, 6-OH).

4.6 References and notes

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5 Diastereoselective Synthesis of Anomeric β-D-Glycosyl Carbamates [1]

In the preceding chapters all synthesized prodrugs possess a β-D-glycosyl carbamate specifier, with the exception of the phosphate- and sulfate-based prodrugs. In the multi-step syntheses of these prodrugs, the glycosyl carbamate moiety is introduced in a very high to nearly 100% β-diastereoselective manner by addition reaction of an anomerically unprotected glycosyl donor to an isocyanate. More detailed mechanistic and synthetic aspects of this reaction will be presented in section 5.1 of this chapter. In section 5.2 a novel β-D-glucuronyl carbamate forming reaction is described.

5.1 Addition of 1-OH of glycosyl donors to isocyanates

5.1.1 Introduction

β-Glucuronyl carbamates of type **1** and **2** (chart 5.1) are well known metabolites of drugs containing a primary [2] or a secondary[3] amino group, respectively. Reports on synthetic methods to prepare these β-D-glucuronyl carbamates, or in a more general sense β-D-glycosyl carbamates of general structure **3**, are very scarce [4].

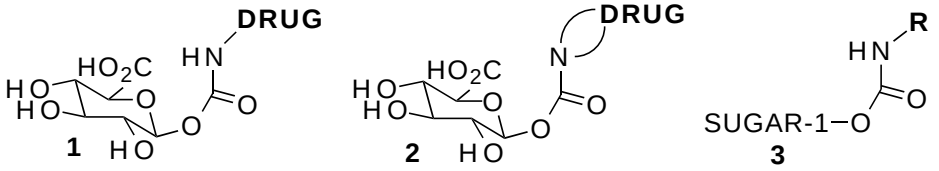
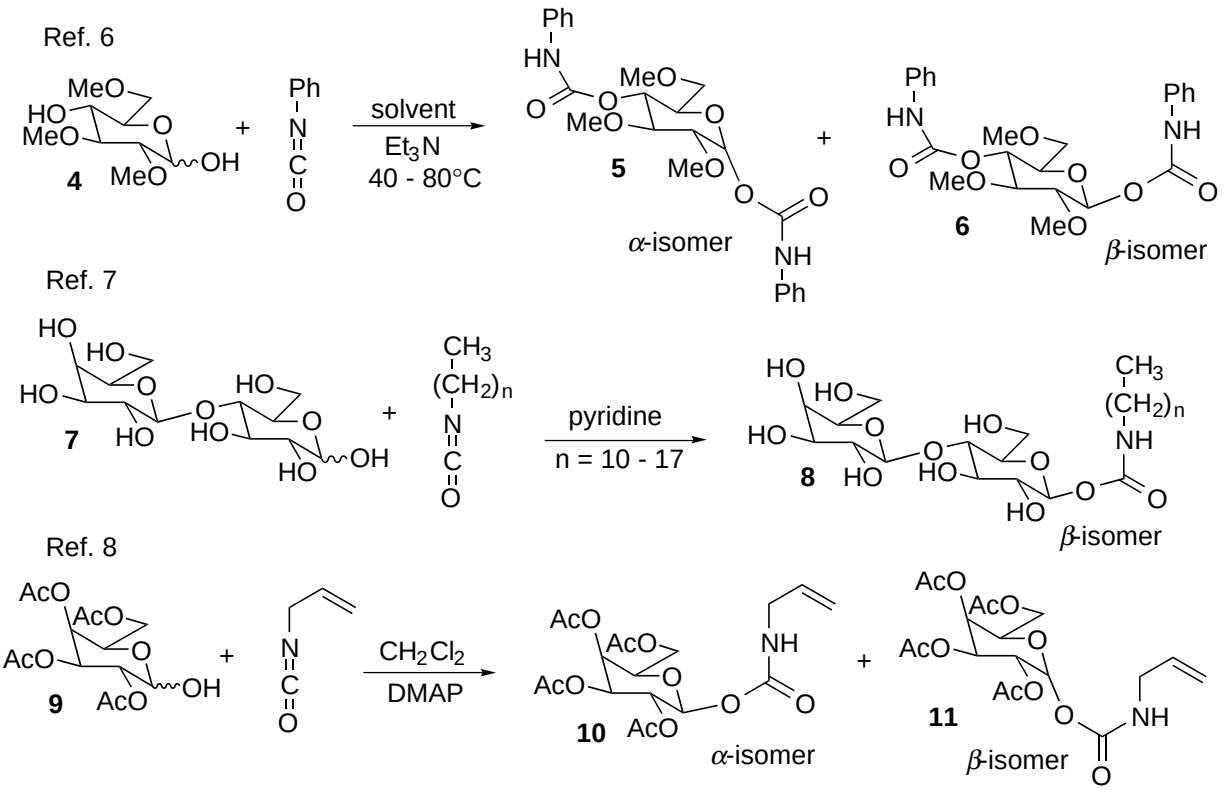


Chart 5.1 *Glycosyl carbamates.*

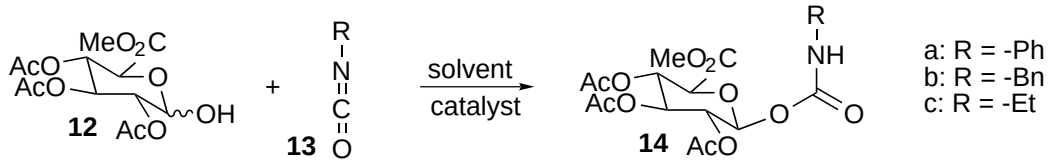
Despite the extensive literature on the addition reaction of alcohols to isocyanates leading to carbamates[5] , only a few examples concerning the addition reaction of the anomeric hydroxyl group of glycosides to isocyanates are described (scheme 5.1). In this context, 2,3,6-tri-*O*-methyl D-glucose **4** and phenyl isocyanate were allowed to react under a variety of conditions resulting in α- and β-dicarbamates **5** and **6**, respectively, with β-diastereoselectivities ranging from 30% to 80% [6]. Unprotected lactose **7** reacted regioselectively with C_{11} - C_{18} alkyl isocyanates to afford 1-*O*-β-lactosyl carbamates **8** in yields between 24% and 41%[7]. Finally, allyl isocyanate was reacted with a variety of carbohydrates, for example with 2,3,4,6-tetra-*O*-acetyl D-galactose **9**, to give the corresponding *N*-allyl 1-*O*-α- and -β-D-galactosyl carbamates **10** and **11** in 84% yield and 80% β-diastereoselectivity [8].



Scheme 5.1 *Literature examples of the addition reaction of the anomeric hydroxyl-group of glycosides to isocyanates.*

5.1.2 Model studies

In order to establish optimal conditions leading to high β-diastereoselectivities (>> 80%), the addition reaction of methyl 2,3,4-tri-*O*-acetyl D-glucuronate **12** and phenyl- benzyl- and ethyl isocyanate (table 5.1, R = -Ph, -Bn and -Et ,respectively) was investigated using different tertiary amines as the catalyst as well as different solvents.



isocyanate	PhMe	PhMe	PhMe	PhMe	CH_2Cl_2	THF
	-	Pyr.	*i*-Pr_2NEt	Et_3N	Et_3N	Et_3N
13a	95	95	>98	100	95	88
13b	88	95	90	>98	88	90
13c †	no reaction		- *	>98		

Addition of **12** (20 mg/mL) to 2.0 equiv. of **13** at 20 °C and 0.3 equiv. of amine.
For reactions in MePh/Et_3N c.y. >90%, determined by NMR.
† Addition of **12** to the **13c** at 60°C.
* Low yield, extensive formation of side products.

Table 5.1 *% β-Diastereoselectivity during formation of **14**.*

The anomeric composition of the products **14** was determined by integration of the clear doublets of H-1(*α*) and H-1(*β*) which were obtained on a 100 MHz FT-NMR from the crude reaction mixture. For details concerning the determination of the anomeric composition, see section 5.1.5. The data in table 5.1 reveal that the addition reaction of **12** to isocyanates **13** in toluene and triethylamine as the catalyst proceeds with optimal *β*-diastereoselectivity. These optimal conditions were employed for the synthesis of the *β*-D-glucuronyl carbamate pro-moieties from glucuronyl donor **12** and a variety of isocyanates. The results are collected in table 5.2. In an analogous manner, using these reaction conditions, 2,3,4,6-tetra-*O*-acetyl D-glucose **18** and 2,3,4,6-tetra-*O*-acetyl D-galactose **19** (chart 5.2) were added to phenyl isocyanate in 100% *β*-diastereoselectivity. Benzyl 2,3,4-tri-*O*-benzyl D-glucuronate **17** reacted with phenylisocyanate in 95% *β*-diastereoselectivity. Reactions of **18** and **19** with other isocyanates resulted in similarly high *β*-diastereoselectivities as obtained for **12**.

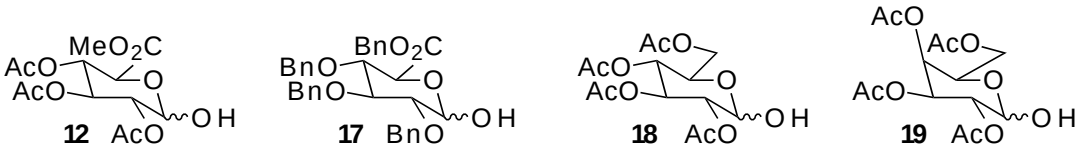
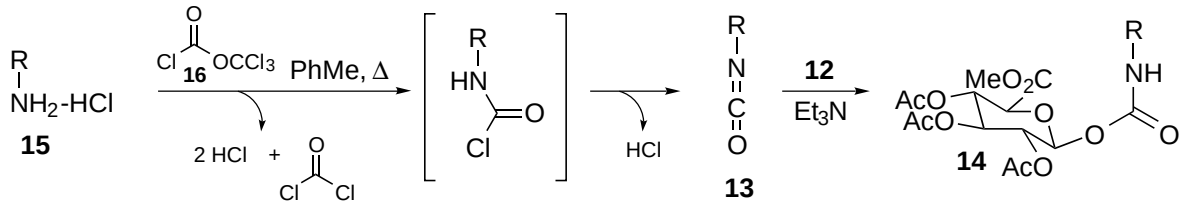


Chart 5.2 *Glycosyl donors.*

5.1.3 Isocyanates from amines and diphosgene

Isocyanates that were not commercially available, were prepared *in situ* from the corresponding amine hydrochlorides **15** and diphosgene (**16**) (scheme 5.2). The liquid diphosgene is a saver alternative for phosgene and is handled more easily than phosgene. After the isocyanate was formed, excess of triethylamine was added to scavenge the liberated HCl and the glucuronyl donor **12** was added to the reaction mixture to furnish *β*-D-glucuronyl carbamates **14**. In a classical fashion, isocyanates can be prepared from amine hydrochlorides using phosgene. The isocyanate is then obtained by distillation of the reaction mixture. When this procedure was followed for the preparation of 2-bromo-4-methylphenyl isocyanate (entry #3 in table 5.2), the isocyanate decomposed during distillation. Decomposition of the isocyanate was circumvented by using the isocyanate without purification.

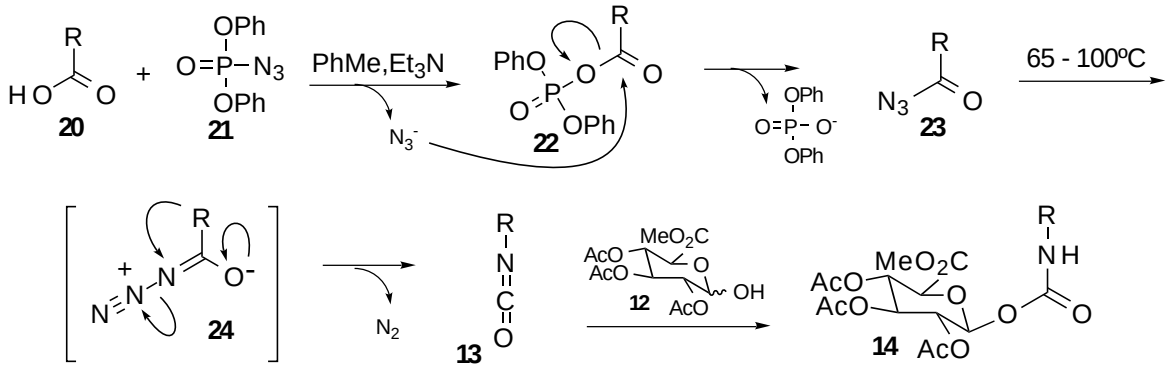


Scheme 5.2 *Preparation of isocyanates from amines-HCl using diphosgene.*

5.1.4 Isocyanates *via* the modified Curtius rearrangement

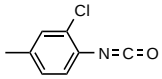
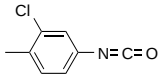
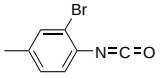
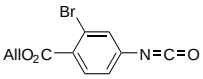
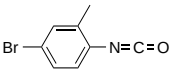
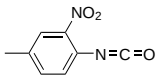
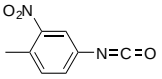
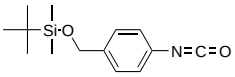
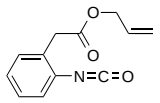
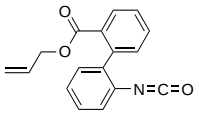
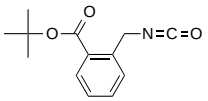
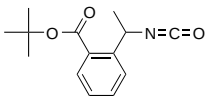
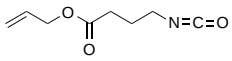
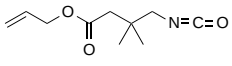
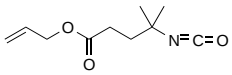
Since a multitude of carboxylic acids are commercially available, the preparation of an isocyanate from a carboxylic acid *via* the Curtius rearrangement gives access to a large number of target compounds.

The rearrangement of acyl azides to isocyanates and molecular nitrogen was discovered more than a century ago by the German scientist Curtius [9]. This ancient reaction can be employed in the synthesis of prodrugs to generate isocyanates **13** from carboxylic acids **20** (scheme 5.3). Using diphenyl phosphoryl azide (**21**) [10], acyl azides **23**[11] were formed from carboxylic acids **20** *via* mixed phosphorus anhydrides **22**[12] . On heating, acyl azides **23** rearrange to isocyanates **13** according to the mechanism depicted in **24**. Diphenyl phosphoryl azide **21** appeared to be an invaluable reagent for a convenient one-pot preparation of acyl azides directly from carboxylic acids. After isocyanates had been formed, the mixture was cooled to ambient temperature (for aromatic and benzylic isocyanates, for aliphatic isocyanates to 50 - 60°C) and anomerically unprotected glucuronate **12** was added to the reaction mixture. Following this protocol, *β*-D-glucuronyl carbamates **14** were obtained in a one-pot procedure from acids **20** in good to excellent yields in more than 95% *β*-diastereoselectivity.



Scheme 5.3 *Modified Curtius rearrangement and further reaction to* ***14****.*

Table 5.2 summarizes the results obtained for the modified Curtius reactions carried out in connection with the synthesis of *β*-D-glucuronyl carbamate containing prodrugs. It was demonstrated by several experiments that reactions of glucosyl donor **18** and galactosyl donor **19** with isocyanates result in similar *β*-diastereoselectivities as obtained for the corresponding *β*-D-glucuronyl carbamates. These results were not included in table 5.2.

entry	isocyanate **13**	general procedure †	rx conditions equiv. Et_3N, T	% β-diastereo-selectivity	% yield	mp of **14** (crystallized from)
#1		2	>1, 20ºC	100	92	142 - 143 (*i*-Pr_2O)
#2		1	0.1, 20ºC	91	95	155 (Et_2O)
#3		2	>1, 0ºC	100	95	139 (*i*-Pr_2O)
#4		3	0.1, 20ºC	>95	80	61 (foam)
#5		1	1.0, 0ºC 1.5, 0ºC 2.0, 0ºC	86 93 100	94	120 - 123 (*i*-Pr_2O/EtOAc)
#6		1	0, 0ºC 0.3, 0ºC 1.0, 0ºC	56 62 100	100	169 (*i*-Pr_2O/EtOAc)
#7		1	1.0 - 3.0, 0ºC	80	100	153 - 156 (*i*-Pr_2O/EtOAc)
#8		3	0.1, 0ºC	100	80	128 (*i*-Pr_2O/*n*-Hex)
#9		3	0.1, 0ºC	100	86	82 (*i*-Pr_2O/EtOAc)
#10		3	0.1, 0ºC	100	89	63 - 67 (*i*-Pr_2O/EtOAc)
#11		3	0.1, 0ºC	>95	68	112 - 119 (Et_2O)
#12		3	0.1, 0ºC	100	62	69 - 74 (Et_2O)
#13		2	0.1, 50ºC	95	74	oil
#14		3	0.1, 60ºC	95	80	30 - 33 foam
#15		3	0.1, 60ºC	100	81	oil

† For the reaction procedure, see the corresponding general procedure # in the experimental section.
1: Commercially available isocyanate; 2: Isocyanate from amine-HCl and diphosgene;
3: Isocyanate from carboxylic acid following the modified Curtius procedure.

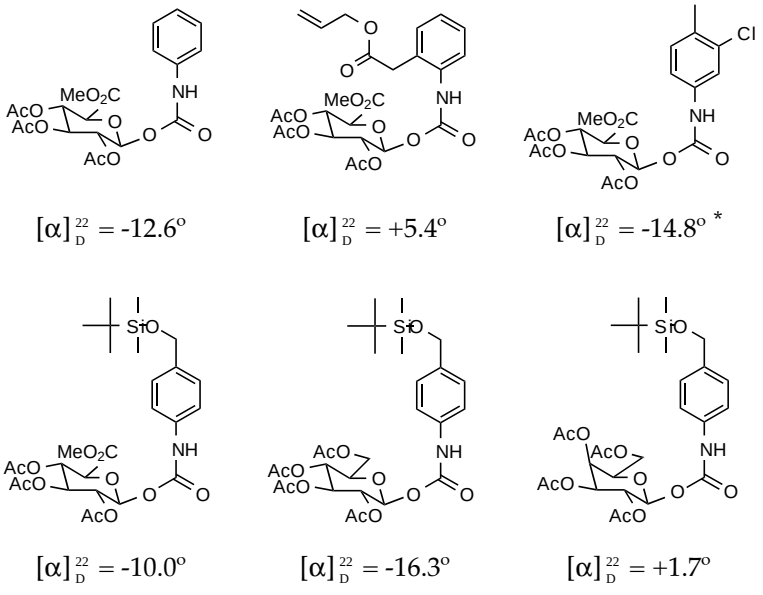
***Table 5.2** β-Diastereoselectivity and yield of addition reaction of methyl 2,3,4-tri-O-acetyl D-glucuronate **12** and isocyanates **13** to β-D-glucuronyl carbamates **14**.*

5.1.5 Determination of the anomeric composition by ^{1}H-NMR

In general, the stereochemical identity of glucuronides at C(1) can easily be deduced from their proton NMR spectra[13] . Both the chemical shift and the coupling constant of the glucuronide H-1 differ for α-D-glucuronides and β-D-glucuronides. In an α-D-glucuronide, typically, δ (1-H$_\alpha$) = 5.2 - 5.5 ppm and the 1-H - 2-H coupling constant (equatorial-axial interaction) is small, J = 2.2 - 2.7 Hz. In a β-D-glucuronide, δ (1-H$_\beta$) = 4.6 - 4.9 ppm and the diaxial coupling constant between 1-H and 2-H is larger, J = 7.3 to 7.9 Hz. For an α-D-glucuronyl carbamate, typically, δ (1-H$_\alpha$) = 6.1 - 6.4 ppm and $J_{1\text{-}2}$ = 2.5 - 2.7 Hz, whereas for a β-D-glucuronyl carbamate δ (1-H$_\beta$) = 5.4 - 5.9 ppm and $J_{1\text{-}2}$ = 7.4 - 8.1 Hz.

Using these characteristics the anomeric composition of the β-D-glucuronyl carbamate products **14** was determined by integration of the clear doublets of H-1(α) and H-1(β) which were obtained from the crude reaction product using a 100 MHz FT-NMR.

In addition, the optical rotation in $CHCl_3$ of a number of representative β-D-glucuronyl carbamate products **14** was determined, see table 5.3.



in all cases c = 1.0 in $CHCl_3$ except: * c = 0.5 in $CHCl_3$

Table 5.3 *Some representative optical rotation values.*

5.1.6 High β-diastereoselectivities

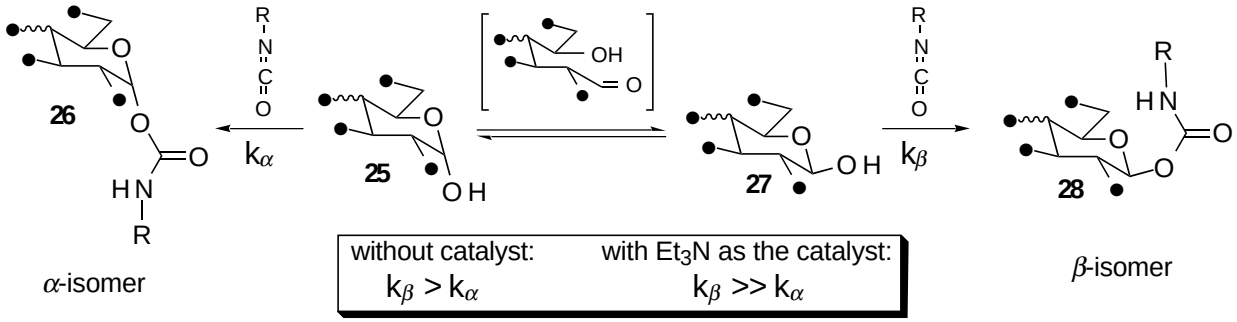
The extremely high β-diastereoselectivities encountered in all addition reactions of the glucuronide-, glucoside- and galactoside anomeric hydroxyl group to isocyanates is clearly no reflection of the anomeric compositions of the respective glycopyranoside hemi acetals in solution. In a number of different organic solvents, the glucuronide hemi acetal **12** appeared[14] to be predominantly in its α-configuration (table 5.4). When triethylamine was added to solutions of **12**, the anomeric composition was even more in favour of the α-isomer as was demonstrated by NMR.

solvent	α/β of **12** in solution
$CDCl_3$ *	4.9/1 (lit.: 100/0 [15], 4/1 [16])
CD_3OD	4/1
MePh-D_8	5/1
THF-D_8	5.1/1

* for **12** and 1 equiv. of Et_3N in $CDCl_3$, only the α-anomer was detected

Table 5.4 *Anomeric composition of **12** in different solvents.*

Despite only approximately one-sixth of the anomeric hydroxyl groups of **12** in toluene are in the β-position (table 5.4), the addition reaction of **12** to isocyanates in the absence a catalyst (table 5.1) results in a high β-diastereoselectivity. The Curtin-Hammett principle, stating that the position of the equilibrium between both anomers **25** and **27** (scheme 5.4) is irrelevant for the product ratio of **26** and **28**, applies to this reaction.



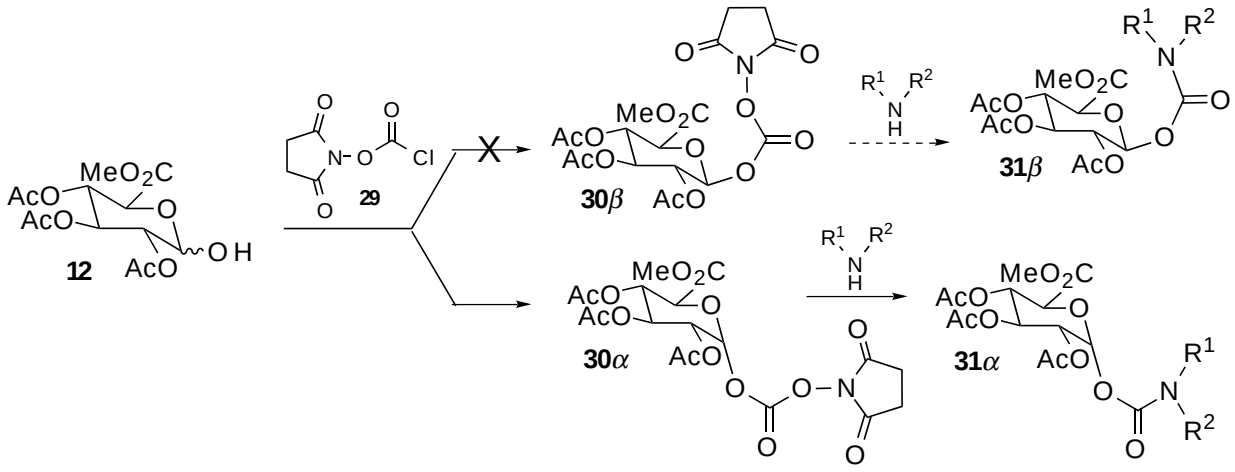
Scheme 5.4 *Curtin-Hammett principle in the β-diastereoselective addition reaction.*

When triethylamine is added to the reaction mixture the β-diastereoselectivity is even more pronounced, implying that the amine catalyzes the formation of the β-product more efficiently than that of the α-product.

The high selectivity probably originates from the 1,3-diaxial steric hindrance in the α-transition state, obstructing the tertiary amine to properly catalyze the addition reaction. Moreover, the 1-OH group is known to be more nucleophilic in the β- than in the α-configuration [17]. Additional evidence supporting the assumption that the β-product formation is more efficiently catalyzed is obtained using very reactive isocyanates. When **12** reacted with 4-methyl-2-nitrophenyl isocyanate (table 5.2) in toluene at 0°C in the absence of a catalyst a mixture of the α- and β-products in a ratio of 1/1 was formed. When the reaction was caried out in the presence of 0.3 equiv. of triethylamine an α/β ratio of 1/2 was obtained. When a stoichiometric amount of triethylamine was used in this reaction, only the β-product was formed (table 5.2). Also in agreement with this supposition is the observation that the use of a sterically hindered amine (diisopropyl ethyl amine, table 5.1) catalyzes the addition reaction less efficiently[5] resulting in lower β-diastereoselectivities.

5.1.7 High α-diastereoselectivities

Alternatively, to prepare α-glucuronyl carbamates, when glucuronyl donor **12** was reacted with *N*-succinimidyl chloroformate (**29**) followed by a reaction of the resulting active carbonate **30** with an amine R^1R^2NH, the α-anomer **31**α was almost exclusively obtained (% of α-anomer > 98, scheme 5.5). Thus, the use of chloroformate **29**[18] provides a synthetic route to α-D-glycosyl carbamates[19].

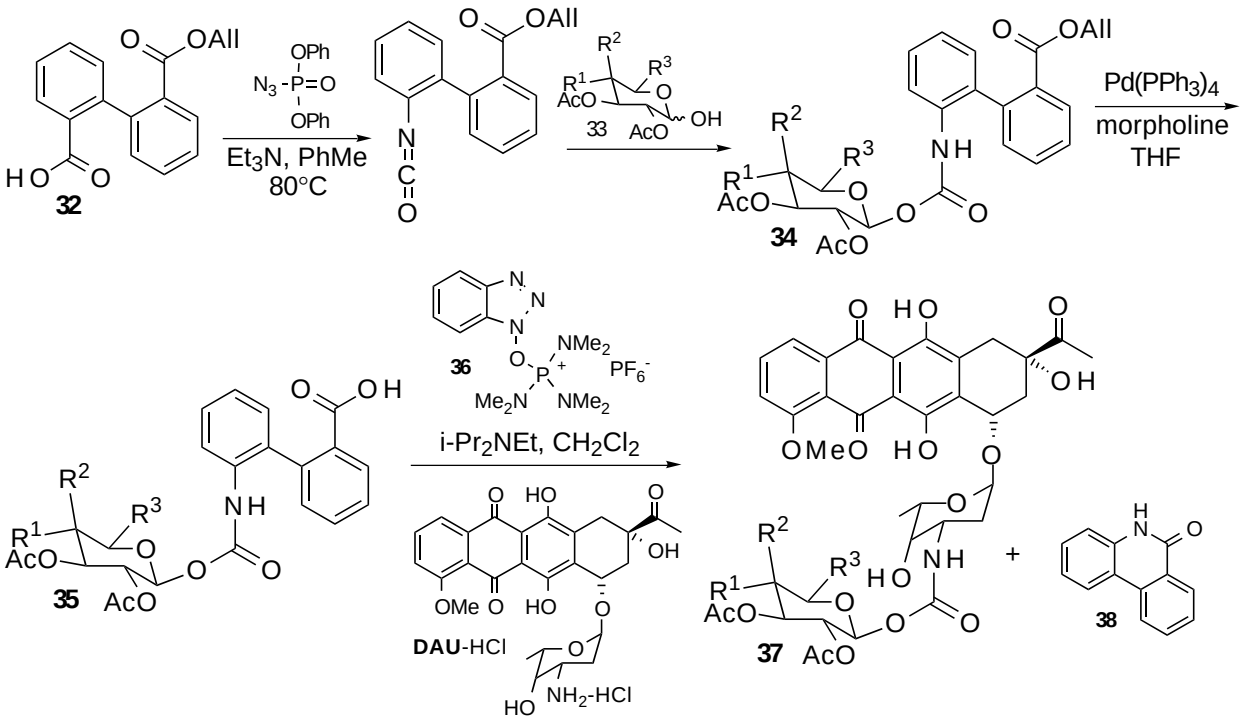


Scheme 5.5 *Preparation of α-D-glucuronyl carbamates using a chloroformate.*

5.2 A novel β-D-glycosyl carbamate forming reaction

5.2.1 Introduction

In an attempt to couple the carboxylic acid group of **35a** to the amino group of daunorubicin (**DAU**) using the BOP coupling reagent **36**, carbamate **37a** was obtained in 89 % together with 6(5*H*)-phenanthridinone (**38**) (scheme 5.6) instead of the expected amide product. This coupling reaction was carried out in an attempt to synthesize **DAU-GB4** (see section 3.3.3 and scheme 3.9). Variation of the peptide coupling reagent, solvent and base did not lead to the desired product; in all cases β-D-glucuronyl carbamate **37a** was isolated. This compound was previously prepared in a more complicated manner during the synthesis of **DAU-GA1** (see section 2.5.2 and scheme 2.11). In the literature, a comparable example of such an intramolecular elimination reaction leading to phenanthridinone **38** has not been reported.

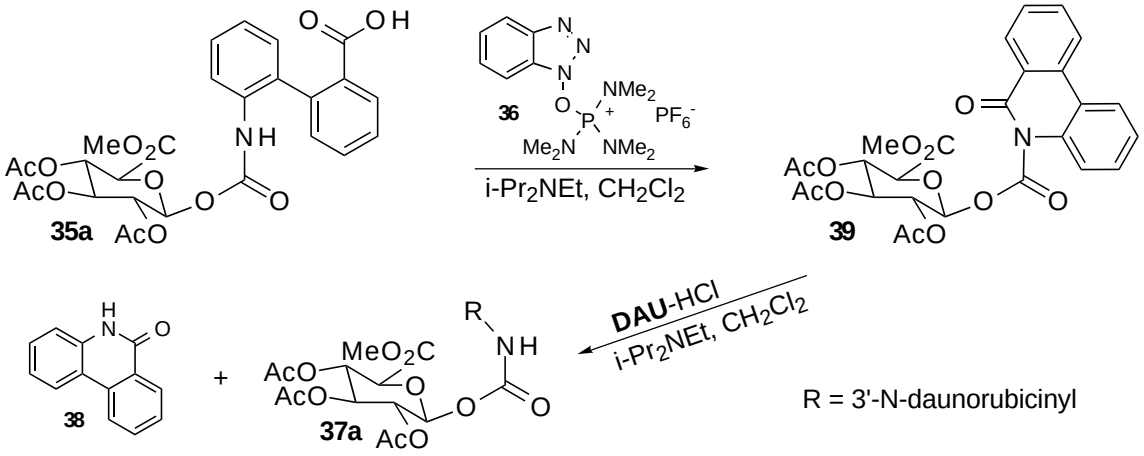


Scheme 5.6 *Transfer of β-D-glucuronyl carbonyl fragment.*

As was mentioned in section 2.5.3, the synthesis of **DAU-GlA1** was accomplished using the galactosyl analog of the reaction in scheme 5.6. In this fashion, compound **37b** was prepared from **35b** in 70% yield. This proved that the novel reaction also worked for the galactosyl analog. Deprotection of **37b** resulted in prodrug **DAU-GlA1**.

The unexpected sequence depicted in scheme 5.6 can be rationalized as follows (see scheme 5.7). The coupling reagent (i.e. BOP) activates the carboxylic acid in the normal manner. Then an intramolecular reaction with the carbamate nitrogen takes place to give the *N*-phenanthridinone O-β-D-glucuronyl carbamate **39**. The phenanthridinone

moiety serves as a leaving group in the subsequent reaction with daunorubicin to give product **37a** and phenanthridinone **38**.



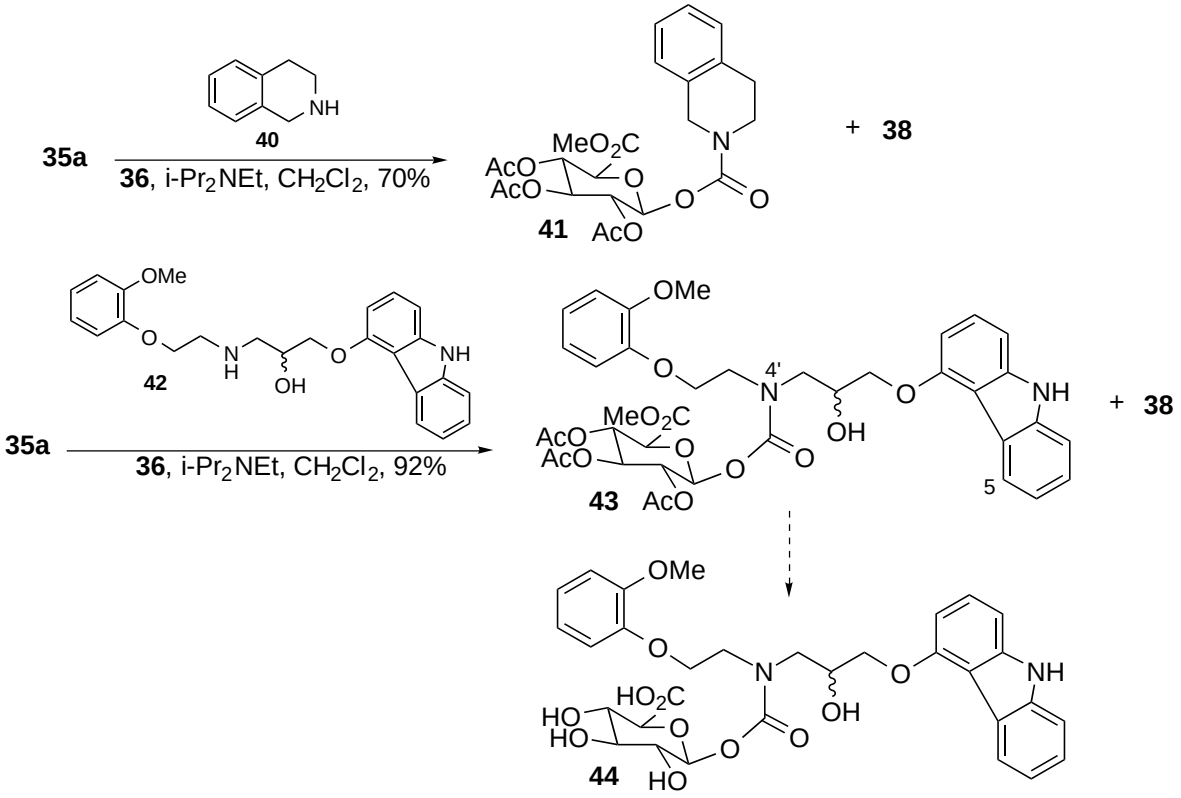
Scheme 5.7 *Cyclization of **32a** and further reaction with amines to form **46**.*

Unequivocal evidence for this mechanism was obtained by a reaction of **35a** with coupling reagent BOP (**36**) in the absence of a nucleophilic amine. This reaction led to the high yield formation of compound **39** (90%). An independent reaction of compound **39** with daunorubicin resulted in carbamate **37a** and phenanthridinone **38**.
It should be noted that the amino group of daunorubicin cannot be converted into an isocyanate group in order to react with a glycosyl donor, because of the presence of the four hydroxyl groups present elsewhere in the molecule which would react with the isocyanate function. Also secondary amines are of interest as a substrate for this novel glycosyl carbamate forming reaction as they principally cannot be transferred into isocyanates.

5.2.2 Application of the novel transfer reaction

Application of the reaction to the synthesis of *β*-D-glucuronyl carbamates of secondary amines was first tested in a model system (scheme 5.8). When 1,2,3,4-tetrahydroisoquinoline **40** was reacted with *N*-2-biphenyl-2'-carboxylic acid *O*-(methyl 2,3,4-tri-*O*-acetyl *β*-D-glucuronyl) carbamate (**35a**, scheme 5.6), the *β*-D-glucuronyl carbamate **41** was obtained in 70% yield. This proved that the reaction also worked for a secondary amine.
As already mentioned in section 5.1.1, *β*-D-glucuronyl carbamates are well known metabolites of drugs containing a primary [2] or a secondary [3] amino group. In this respect, in rats the *β*-D-glucuronyl carbamate **44** (scheme 5.7) was found to be a metabolite of the anti-hypertensive drug carvedilol **42** [20]. The metabolite had been prepared for use as a reference compound *in vitro* using dog and rat liver microsomes [21]. Using the novel *β*-D-glucuronyl carbonyl transfer reaction compound **43** could be prepared in 92% yield from (±)-carvedilol **42**. This is the protected precursor of metabolite **44**. The preparation of *N*-daunorubicinyl- and *N*-carvediloyl *O*-*β*-D-glucuronyl carbamates **37a** and **43**, respectively, exemplifies the value of the

reaction in the preparation β-D-glucuronyl carbamates of relatively complicated primary and secondary amines.



Scheme 5.8 *β-Glucuronyl carbamates of secondary amines.*

5.3 Experimental part

General

Daunorubicin hydrochloride was a generous gift of PharmaChemie BV (Haarlem, The Netherlands), (±)-carvedilol was a gift of Boehringer Mannheim GmbH (Mannheim, Germany). ^{1}H-NMR spectra at 100 MHz were obtained on a Bruker AM-100 spectrometer, spectra at 400 MHz on a Bruker AM-400 spectrometer. Chemical shifts are expressed in ppm downfield from internal standard Me_4Si. In all cases, CH_2Cl_2, Et_3N and PhMe were dried by distillation over CaH_2 and *i*-Pr_2NEt was dried over KOH pellets.

General procedure #1: Reaction of glucuronyl donor 12 with commercially available isocyanates 13 to obtain β-D-glucuronyl carbamates 14 (scheme 5.2).

To a solution of 200 mg of isocyanate **13** and 0.1 to 2.0 equiv. (see table 5.2) of Et_3N in 20 mL of dry PhMe, 0.5 equiv. of glycosyl donor **12** was added. After 2 h, the reaction mixture was concentrated to dryness and subjected to flash column chromatography (SiO_2, Et_2O/*n*-Hex) to yield 1-*O*-*β*-(methyl 2,3,4-tri-*O*-acetyl)-D-glucuronyl carbamates **14** in good to excellent yield and in 95-100% *β*-diastereoselectivity. In most cases the pure *β*-isomer was crystallized (see table 5.2).

General procedure #2: Reaction of glucuronyl donor 12 with isocyanates *in situ* prepared from an amine-HCl 15 and diphosgene 16 to obtain *β*-D-glucuronyl carbamates 14 (scheme 5.2).

200 mg of amine-HCl **15** and 1.0 equiv. of diphosgene (**16**) were dissolved in 10 mL of PhMe and the reaction mixture was refluxed for 2 h under an argon atmosphere. When the HCl evolution had ceased, the mixture was cooled to 0ºC and 5.0 equiv. of Et_3N were added. After that, 0.5 equiv. of **12** was added. The reaction mixture was stirred for 1 h at 0ºC and processed according to general procedure #3. The crude product was purified by column chromatography (SiO_2, Et_2O/*n*-Hex 1/1) to give 1-*O*-*β*-(methyl 2,3,4-tri-*O*-acetyl)-D-glucuronyl carbamates **14** in good to excellent yield and in 95-100% *β*-diastereoselectivity. In most cases the pure *β*-isomer was crystallized (see table 5.2).

General procedure #3: Modified Curtius reaction for conversion of carboxylic acids 20 to *β*-D-glucuronyl carbamates 14 (scheme 5.3).

500 mg of a carboxylic acid **20** was stirred overnight with 1.1 equiv. of $(PhO)_2P(O)N_3$ (**21**) and 1.1 equiv. of Et_3N in 15 mL of PhMe under an argon atmosphere at room temperature. The reaction mixture was subsequently heated until gas evolution was observed, occurring between 65-100ºC depending on the actual substrate. Heating was continued for 2 h. After that, for aromatic and benzylic isocyanates the reaction mixture was allowed to cool to ambient temperature. Aliphatic isocyanates were allowed to cool down to 50-60ºC. 0.5-0.75 equiv. [22] of the glucuronate **12** was added. The reaction mixture was stirred until **12** had almost disappeared on TLC (SiO_2, Et_2O) and worked-up in the following fashion: The reaction mixture was diluted with 100 mL of Et_2O and successively washed with 200 mL portions of aqueous 0.5 N $KHSO_4$, with saturated aqueous $NaHCO_3$ (3x) and with brine. The organic layer was dried over Na_2SO_4 and evaporated. The residual material was purified by column chromatography (SiO_2, Et_2O/*n*-Hex) to yield the 1-*O*-*β*-(methyl 2,3,4-tri-*O*-acetyl)-D-glucuronyl carbamates **14** in good to excellent yield and in 95-100% *β*-diastereoselectivity. In most cases the pure *β*-isomer was crystallized (see table 5.2).

General procedure #4: Preparation of *β*-D-glucuronyl carbamates of amines using the novel *β*-glycosyl carbamate forming reaction.

100 mg of **35a** [23], 1.1 equiv. of BOP (**36**), 3 equiv. of *i*-Pr_2NEt and 1 - 2 equiv. of the amine-substrate were dissolved in 20 mL of CH_2Cl_2 and stirred overnight under an argon atmosphere at ambient temperature. The course of the coupling reaction was monitored on TLC (SiO_2, Et_2O). After that, the reaction mixture was diluted with 150 mL of CH_2Cl_2 and successively washed with 200 mL portions of aqueous 0.5 N $KHSO_4$ (3x), saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4 and evaporated. The residual material was purified by column chromatography (SiO_2 Et_2O/*n*-Hex or CH_2Cl_2/EtOH) to yield the respective 1-*O*-*β*-(methyl 2,3,4-tri-*O*-acetyl)-D-glucuronyl carbamates in good to excellent yield and in 100% *β*-diastereoselectivity (the starting material **35a** was 100% *β*-anomer, see entry #10 in table 5.2. No anomerization was detected after completion of the reaction).

Synthesis of *N*-3'-daunorubicinyl *O*-*β*-(2,3,4-tri-*O*-acetyl D-glucuronyl) carbamate (37a scheme 5.6).

According to general procedure #4 in 89%. For the physical data of **37a**, see the experimental part of chapter 2 where this compound is prepared differently [compound **38*β*** under "synthesis of *N*-3'-daunorubicinyl *O*-*β*-D-glucuronyl carbamate (**DAU-GA1**)"]. For this alternative synthesis, see section 2.5.2 and scheme 2.11.

Synthesis of *N*-3'-daunorubicinyl *O*-*β*-(2,3,4,6-tetra-*O*-acetyl D-galactosyl) carbamate (37b scheme 5.6).

N-2-[Biphenyl-2'-carboxylic acid allyl ester] O-β-(2,3,4,6-tetra-O-acetyl D-galactosyl) carbamate (**34b**) from **33b** and **32** (scheme 5.6) according to general procedure #3 in 65% yield and 100% *β*-diastereoselectivity as an oil. In the ^{1}H-NMR spectrum of **34b** taken on a 100 MHz recorder in $CDCl_3$, two ArN*H*- signals were present (in a ratio close to 1/1) and almost all other signals were broadened. This was also seen in the NMR spectrum of the *β*-D-glucuronyl analog **34a** and indicated the existence of two rotameric forms of the compound. This is likely due to restricted rotation around the Ar-Ar bond. ^{1}H-NMR (100 MHz,

$CDCl_3$) δ (ppm) = 1.91, 1.96, 1.98 and 2.10 (4s, 12H, 4 -OAc), 3.95-4.15 (m, 3H, Gal5,6-H_2), 4.44 (bd, 2H, -OCH_2CH=, *J* = 5.6 Hz), 4.90-5.25 (m, 4H, =CH_2 Gal2,3-H), 5.34 (d, 1H, Gal4-H, *J* = 3.0 Hz), 5.50-5.85 (m, 1H, -CH=), 5.62 (d, 1H, Gal1-H, *J* = 7.7 Hz), 6.45 and 6.49 (2s, 1H, ArN*H*-), 7.00-7.65 (m, 6H, Ar3,3',4,4',5,5'-H), 7.70-8.00 (m, 2H, Ar6,6'-H).

N-2-[Biphenyl-2'-carboxylic acid] O-β-(2,3,4,6-tetra-O-acetyl D-galactosyl) carbamate (**35b**) from **34b** according to general procedure #3 of chapter 3 in 93% as an oil.

N-3'-Daunorubicinyl O-β-(2,3,4,6-tetra-O-acetyl D-galactosyl) carbamate (**37b**) from **35b** analogously to general procedure #4 (**35b** is used instead of **35a**) in 70%, mp 160-162ºC, ^{1}H-NMR (400 MHz, $CDCl_3$) δ (ppm) = 1.30 (d, 3H, 5'-Me, *J* = 6.4 Hz), 1.79 (dt, 1H, 2'$_{ax}$-H, *J* = 13.0 Hz *J* = 4.2 Hz), 1.90 (dd, 1H, 2'$_{eq}$-H, *J* = 13.0 Hz *J* = 5.0 Hz), 1.98 (s, 6H, 2 -OAc), 2.04, and 2.14 (2s, 6H, 2 -OAc), 2.11 (dd, 1H, 8$_{ax}$-H, *J* = 14.8 Hz *J* = 4.0 Hz), 2.31 (d, 1H, 8$_{eq}$-H, *J* = 14.8 Hz), 2.42 (s, 3H, 9-C(O)Me), 2.48 (s, 1H, 4'-OH), 2.90 (d, 1H, 10$_{ax}$-H, *J* = 18.8 Hz), 3.22 (d, 1H, 10$_{eq}$-H, *J* = 18.8 Hz), 3.65 (d, 1H, 4'-H, *J* = 7.0 Hz), 3.88 (bs, 1H, 3'-H), 4.07 (s, 3H, 4-OMe), 3.95-4.15 (m, 3H, Gal5,6-H_2) 4.22 (q, 1H, 5'-H, *J* = 4.22 Hz), 4.45 (s, 1H, 9-OH), 5.05 (dd, 1H, Gal3-H, *J* = 10.4 Hz *J* = 3.3 Hz), 5.20-5.35 (m, 3H, 3'-NH- 7-H Gal2-H), 5.39 (d, 1H, Gal4-H, *J* = 3.7 Hz), 5.50 (d, 1H, 1'-H, *J* = 3.5 Hz), 5.58 (d, 1H, Gal1-H, *J* = 8.3 Hz), 7.38 (d, 1H, 3-H, *J* = 8.4 Hz), 7.77 (t, 1H, 2-H, *J* = 8.4 Hz), 8.02 (d, 1H, 1-H, *J* = 7.7 Hz), 13.24 (s, 1H, 11-OH), 13.97 (s, 1H, 6-OH).

Synthesis of *N*-2-(1,2,3,4-tetrahydroisoquinolinyl) *O*-β-(methyl 2,3,4-tri-*O*-acetyl D-glucuronyl) carbamate (41 scheme 5.8).

According to general procedure #4 in 70% (calculated from **35a**) from **35a** and 1,2,3,4-tetrahydroisoquinoline (**40**) (2.0 equiv.) as an oil. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.90-2.10 (m, 2H, 4-H_2), 2.04 (s, 9H, 3 -OAc). 2.65-3.00 (m, 2H, 3-H_2), 3.72 (s, 3H, -CO_2Me), 4.22 (d, 1H, Gluc5-H, *J* = 9.3 Hz), 4.51 (d, 1H, 1-H_aH$_b$, *J* = 12.1 Hz), 4.68 (d, 1H, 1-H$_a$$H_b$, *J* = 12.1 Hz), 5.15-5.50 (m, 3H, Gluc2,3,4-H), 5.76 (d, 1H, Gluc1-H, *J* = 7.0 Hz), 7.05-7.30 (m, 4H, 4Ar-H).

Synthesis of *N*-4'-carvediloyl *O*-β-(methyl 2,3,4-tri-*O*-acetyl D-glucuronyl) carbamate (43 scheme 5.8).

According to general procedure #4 in 92% (calculated from **42**) from **35a** and (±)-carvedilol (**42**) (0.9 equiv.), mp 58ºC, MS (FAB$^+$) *m*/*z* = 789 ([M+Na]$^+$), 766 ([M]$^+$), 317 ([$C_{13}H_{17}O_9$]$^+$). Because a racemic mixture of carvedilol was used, two diastereomeric carbamoyl glucuronides were obtained. This resulted in overlapping and broadening of signals in the proton NMR. ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 1.90, 1.93, 1.95, 1.97 and 2.03 (5s, 9H, 3 -OAc), 3.40-5.20 (m, 9H, aliphatic carvedilol), 3.73 (s, 6H, -OMe -CO_2Me), 4.25 (bd, 1H, Gluc5-H, *J* = 9.2 Hz), 5.10-5.50 (m, 3H, Gluc2,3,4-H), 5.79 (bd, 1H, Gluc1-H, *J* = 7.1 Hz), 6.60-7.60 (m, 10H, 10 Ar-H), 8.09 and 8.12 (2s, 1H, ArN-*H*), 8.20-8.40 (m, 1H, Ar5-H).

***N*-6-Phenanthridinone-5-yl *O*-β-(methyl 2,3,4-tri-*O*-acetyl D-glucuronyl) carbamate (39 scheme 5.7).**

According to general procedure #4 (no amine was added) in 90% yield from **35a** as an oil, ^{1}H-NMR (100 MHz, $CDCl_3$) δ (ppm) = 2.04, 2.07 and 2.20 (3s, 9H, 3 -OAc), 3.80 (s, 3H, -CO_2Me), 4.35 (d, 1H, Gluc5-H, *J* = 9.3 Hz), 5.15-5.55 (m, 3H, Gluc2,3,4-H), 6.08 (d, 1H, Gluc1-H, *J* = 7.6 Hz), 7.10-8.35 (m, 7H, Ar3,3',4,4',5,5',6'-H), 8.39 (dd, 1H, Ar6-H, *J* = 7.8 Hz *J* = 1.5 Hz). MS (FAB$^+$) *m*/*z* = 578([M+Na]$^+$), 317 ([$C_{13}H_{17}O_9$]$^+$).

5.4 References and notes

[1] Part of section 5.1 has been published: Leenders, R.G.G.; Ruytenbeek, R.; Damen, E.W.P.; Scheeren, J.W. Highly Diastereoselective Synthesis of Anomeric *O*-*β*-Glycopyranosyl Carbamates from Isocyanates. *Synthesis*, **1996**, 1309-1312. Part of section 5.2 is submitted for publication to *Tetrahedron Lett.*: Leenders, R.G.G.; Scheeren, J.W.; A Novel 1-*O*-*β*-Glycosyl Carbamate Forming Reaction.

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[14] The anomeric composition of **12** in different solvents was determined using the respective signals of H-5(*α*) and H-5(*β*) which were obtained on a 100 MHz FT-NMR. The H-1(*α*) and H-1(*β*) signals could not be used because of overlap with other signals.

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[16] a] Nudelman, A.; Herzig, J.; Gottlieb, H.E. *Carbohydr. Res.* **1987**, *162*, 145; b] Tietze, L.F.; Seele, R. *Carbohydr. Res.* **1986**, *148*, 349.

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[18] Using *p*-nitrophenyl chloroformate, a similar *α*-diastereoselectivity was obtained. Using di-*N,N'*-succinimidyl carbonate (see section 4.3.2, scheme 4.5, compound **25**) to prepare active carbonate **37**, no reaction took place.

[19] When 2,3,4,6-tri-*O*-acetyl *β*-glucose (**18**, chart 5.2) instead of methyl 2,3,4-tri-*O*-acetyl glucuronic acid **12** was used in the reaction sequence depicted in scheme 5.5, the *β*-product was obtained in 90% diastereoselectivity (see section 2.5.3 and scheme 2.12). This was due to the fact that the pure 1-*β*-OH anomer was used which was crystallized from the anomeric mixture.

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[22] Less than a stoichiometric amount of **12** is added because a fraction of the *in situ* formed isocyanate dimerizes. In case of the reaction in entry #9 of table 5.2, 22 % of the symmetrical urea was isolated after completion of the reaction.

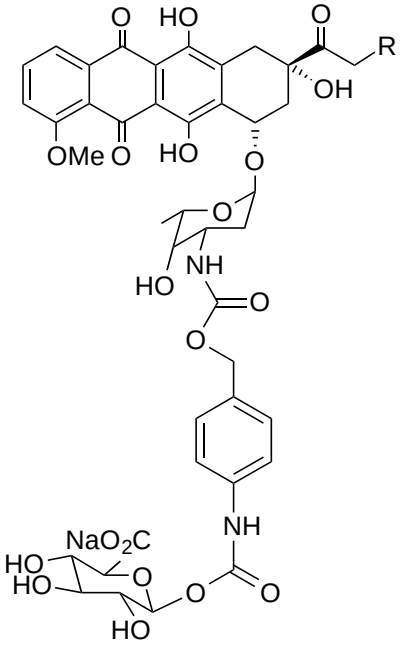
[23] For the synthesis and physical data of **35a**, see the experimental part of chapter 3, compound **50b** which is given under "Attempted synthesis of *N*-2-[2'-(daunorubicin-3'-*N*-carbonyl)]biphenyl *O*-*β*-D-glucuronyl carbamate sodium salt (**DAU-GB4**)".

6 Evaluation of the β-Glucuronide Based ADEPT System for Clinical Application

6.1 Introduction

The practical application of ADEPT in patients suffering from a malignant disease requires multidisciplinary research. In this perspective a collaboration was set-up between the synthesis group of the University of Nijmegen and the Department of Medical Oncology, Vrije Universiteit Hospital of Amsterdam (Dr. H.J. Haisma, Dr. E. Boven). Within the framework of the PhD research of Ir. P.H.J. Houba, this group investigated the preparation of a suitable mAb-β-glucuronidase conjugate and the biological and biochemical aspects of the ADEPT concept.

In our approach toward the development of an effective anti-cancer drug treatment according to the ADEPT concept, a number of stages can be discerned. an enzyme in combination with a prodrug prototype was selected that met specific requirements (see section 1.4). The selection of a suitable ADEPT system was outlined in chapter 2. This resulted in the choice of human β-glucuronidase as the enzyme in combination with a β-D-glucuronide containing prodrug. In stage two (chapters 3 and 4) prodrugs were synthesized which were screened initially with respect to the rate of activation to the parent drug by human β-glucuronidase and with respect to the prodrug cytotoxicity. In subsequent research the pharmacokinetics (e.g. plasma clearance half-life) of a number of selected prodrugs were studied and derivatives of these prodrugs were synthesized in order to optimize these pharmacokinetics. As a result of these investigations one daunorubicin and one doxorubicin prodrug, **DAU-GA3** and **DOX-GA3** (chart 6.1) respectively, were selected as the most promising prodrugs in ADEPT. The synthesis, activation rates and toxicities of these prodrugs were reported in chapter 4.



DAU-GA3 (R = -H)
DOX-GA3 (R = -OH)

Chart 6.1

During the development of a suitable prodrug for ADEPT described in this thesis, research focusing on the preparation of a mAb-enzyme conjugate was undertaken at the department of Medical Oncology, Vrije Universiteit Hospital of Amsterdam. Subsequent investigations in this group were devoted to the development of ADEPT *in vitro* (stage 2) and *in vivo* (stage 3). These investigations will be presented in the thesis of

Ir P.H.J Houba [1] and have been partly published already [3, 4]. A brief summary of this work is given in the subsequent sections.

6.2 The mAb-enzyme conjugate

6.2.1 Monoclonal antibody

For the preparation of mAb-enzyme conjugates mAb 323/A3 was used. This mAb is reactive with antigens highly expressed on the surface of most carcinomas including breast, ovary, lung and colon tumors. Breast cancer and ovarian cancer are considered to be sensitive to anthracyclines.

6.2.2 Enzyme

Human β-glucuronidase (GUS) was chosen for use in ADEPT for the following reasons. First, GUS is localized intracellularly in microsomes and lysosomes. Enzyme activity is not detectable in human blood and premature activation of a β-D-glucuronide containing prodrug is minimized because the hydrophilic prodrugs do not enter cells. Second, β-D-glucuronide containing prodrugs could be prepared which met some essential primary requirements for prodrugs in ADEPT, viz. *i.* stability under physiological conditions, *ii.* reduced toxicity compared with the parent drug and *iii.* rate of activation to the parent drug. Third, GUS was chosen because it is of human origin and therefore, it is expected to be less immunogenic than enzymes derived from other species than man [2].

GUS was produced by a mouse cell line (L-MPR cells) transfected with the human β-glucuronidase gene and secreted in the culture medium from which it was isolated by ion exchange chromatography.

A disadvantage of the use of GUS in ADEPT is the low pH-optimum of 4.2. The enzyme has an approximately 10-fold lower rate of hydrolysis at pH = 6.8 found in the interstitium of tumor tissue. Another disadvantage is that GUS has a rapid glycan-specific hepatic clearance from the blood and is accumulated in the liver; hepatic receptors recognize specific sugar moieties on the enzyme surface. GUS was cleared from the circulation of mice after intravenous administration with a half-life of 30 min. The mAb 323/A3-GUS conjugate showed a half-life even faster than that of native GUS, possibly making it ineffective for use in ADEPT. To decrease the clearance rate of the enzyme, the carbohydrate groups of GUS were modified by subsequent treatment of GUS with $NaIO_4$ and with $NaBH_4$. The enzyme activity of the resulting modified GUS was not decreased by these chemical reactions.

6.2.3 mAb-enzyme conjugate

Modified GUS (mGUS) was coupled to mAb 323/A3 and the clearance rate of this conjugate in mice was greatly improved to a half-life of 8.6 h. The conjugate retained >95% of the GUS activity and also >95% of the immunoreactivity. A radioactive [^{125}I]-323/A3-mGUS conjugate was prepared to study the localization of the conjugate in tumor and other tissue. After coupling the conjugate was purified using a size

exclusion column which separates molecules with different molecular weight. The preparation of these conjugates was published [3] in 1996.

6.3 *In vitro* experiments

6.3.1 Characterization of prodrugs

The GUS mediated activation was determined for all anthracycline prodrugs which were synthesized and described in chapters 2, 3 and 4. In this context, K_m and V_{max} values were calculated and the half-life of the reaction was determined. In this thesis, the prodrug activation rates were given at the end of each respective chapter and were expressed as half-lives ($t_{1/2}$) determined under standard conditions (100 μM Prodrug in 0.1% BSA/PBS, 0.03 U/mL GUS, pH = 6.8, 37ºC). Cytotoxicities of prodrugs which were activated to their parent drug at an agreeable rate were determined. In most cases where a prodrug could not be enzymatically activated to the parent drug, determination of the cytotoxicity was omitted. A comprehensive overview of enzymatic prodrug activation characteristics and cytotoxicities will be described in the thesis of Ir Houba [1]. An evaluation of four prodrugs for use in ADEPT described in this thesis [**DAU-GA1** (Ch. 2), **DAU-GB1** and **DAU-GB5** (Ch. 3), **DAU-GA3** (Ch. 4)] and of two prodrugs prepared by others has been published [4]. In addition to the prodrug activation rate and cytotoxicity, the hydrophilicity, protein binding and cellular uptake of the prodrugs were determined. Based on these results prodrugs **DAU-GA3** and **DOX-GA3** (chart 6.1) were chosen as most promising to be used in ADEPT.

6.3.2 *In vitro* efficacy of ADEPT

Pretreatment of OVCAR-3 cells with the mAb 323/A3-mGUS conjugate and removal of the non-bound conjugate followed by the addition of prodrug **DAU-GA3** to the cells, resulted in a cytotoxicity reaching the same value as that for treatment of daunorubicin alone. When OVCAR-3 cells were preincubated with excess of mAb followed by exposure to the mAb-enzyme conjugate, prodrug **DAU-GA3** was not activated to daunorubicin. These results indicate the specificity of the conjugate. When prodrug was added 24 h after treatment of the OVCAR-3 cells with mAb 323/A3-mGUS, no reduction in cytotoxicity was found. This indicates that the enzyme activity of the bound conjugate is retained, that the conjugate is not internalized by the cells and that shedding of the antigen did not take place (shedding is release of antigen from the cell).

6.4 *In vivo* experiments; animal models

6.4.1 *In vivo* characterization of prodrug and mAb-enzyme conjugate

The elimination of **DAU-GA3** from the circulation of BALB/c mice was determined at a dose of 10 and 100 mg/kg. In both cases an elimination half-life time of 20 min was found. Daunorubicin was eliminated from the circulation with a half-life of 720 min. These results are comparable to those of **DOX-GA3** and doxorubicin. The maximum tolerated dose (MTD) in mice of **DAU-GA3** and **DOX-GA3** was found to be 2x 250 and 2x 500 mg/kg, respectively, (7 days between first and second dose). Compared to

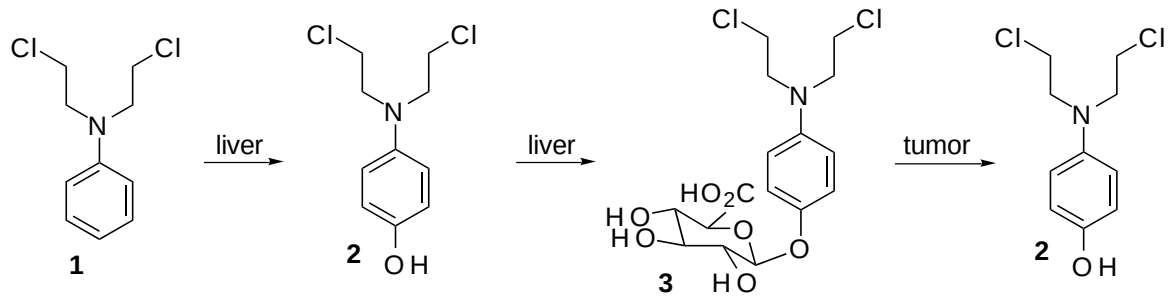
daunorubicin (MTD = 2x 10 mg/kg) and doxorubicin (MTD = 2x 8 mg/kg) these prodrugs were much less toxic.
The pharmacokinetics in BALB/c mice of 323/A3-mGUS and [^{125}I]-323/A3-mGUS conjugates were found to be identical. Blood $t_{1/2}$ = 8.6 h were found for both conjugates. The distribution and tumor localization of [^{125}I]-323/A3-mGUS in OVCAR-3 (human ovarian cancer) xenografted nude mice were determined. At ten days after administration of the conjugate the tumor to blood ratio was >4. The tumor to tissue ratios rose steadily for all organs indicating the specific retention of the conjugate into the tumor and reached a maximum value of >5 at 7 days.

6.4.2 ADEPT

In an *in vivo* ADEPT experiment, mAb 323/A3-mGUS was given to nude mice bearing ovarian cancer xenografts. After 7 days, 50, 100 or 250 mg/kg **DAU-GA3** was given to the mice. A delay in tumor growth was observed for the mice which had received 100 or 250 mg/kg prodrug. The tumor growth delay was more pronounced than that in mice which were conventionally treated with daunorubicin. These first experiments indicate the feasibility of ADEPT in a mouse model.

6.4.3 Monotherapy

Observations made by Connors *et al.* as early as 1966 [5] revealed that two types of plasma cell tumors grown in BALB/c mice were highly sensitivity towards aniline mustard **1** (scheme 6.1). In further experiments to investigate the surprisingly high selective toxicity of aniline mustard **1** for these cancer cells, it was found that **1** is hydroxylated in the liver to the highly toxic hydroxyaniline mustard **2** and then detoxified by conversion to the *β*-D-glucuronide **3**. Additionally, an increased *β*-glucuronidase activity was found in the two plasma cell tumors and a direct relationship was observed between the *β*-glucuronidase activity and the sensitivity to **1**. It was concluded that the highly toxic hydroxyaniline mustard **2** is selectively activated in those tumors by the elevated levels of endogenous *β*-glucuronidase. It should be noted that this high *β*-glucuronidase activity of various cancer tissues has already been reported in 1947 [6].



Scheme 6.1 *Glucuronidation/deglucuronidation of aniline mustard **1**.*

In a more recent paper, Bosslet *et al.* [7] (surprisingly *not* referring to the above-mentioned findings of Connors and co-worker) have reported that high *β*-glucuronidase activities were found especially in necrotic tumor areas where dying

cells liberate their lysosomal β-glucuronidase. This was demonstrated for various human bioptics viz. for breast cancer, lung cancer, gastrointestinal tract cancer and melanomas. It was hypothesized that tumors with a diameter >3 mm develop necrosis primarily due to poor vascularization. As a consequence, the cell contents of necrotic cells, including lysosomal β-glucuronidase, is liberated in those areas. In this study of Bosslet and co-workers it was stated that the released β-glucuronidase is much more resistant toward proteases present in the plasma than other enzymes released by defective cells and, consequently, high β-glucuronidase activities are present in necrotic tumor areas. As already suggested by Connors *et al.* in 1966 [5], Bosslet and co-workers suggested [7] that the β-glucuronidase activity in these necrotic tumor areas can be used to activate a β-D-glucuronide containing prodrug to result in a more tumor selective chemotherapy. In a recent study of Bosslet and co-workers in collaboration with other groups [8], it was shown in a monotherapy experiment using a doxorubicin prodrug (compound **10** chart 4.2) in a human lung tumor model that doxorubicin was accumulated in the tumor cells. This was ascribed to the elevated β-glucuronidase activity of the lung tumor which activated the prodrug.

In the context of these findings and suggestions, further experiments using **DAU-GA3** and **DOX-GA3** without the administration of a mAb-enzyme conjugate were undertaken. Nude mice bearing human ovarian cancer xenografts (OVCAR-3) were used for monotherapy experiments with **DAU-GA3** and **DOX-GA3**. These tumors had a size that necrosis could be expected and, therefore, will display an enhanced β-glucuronidase activity.

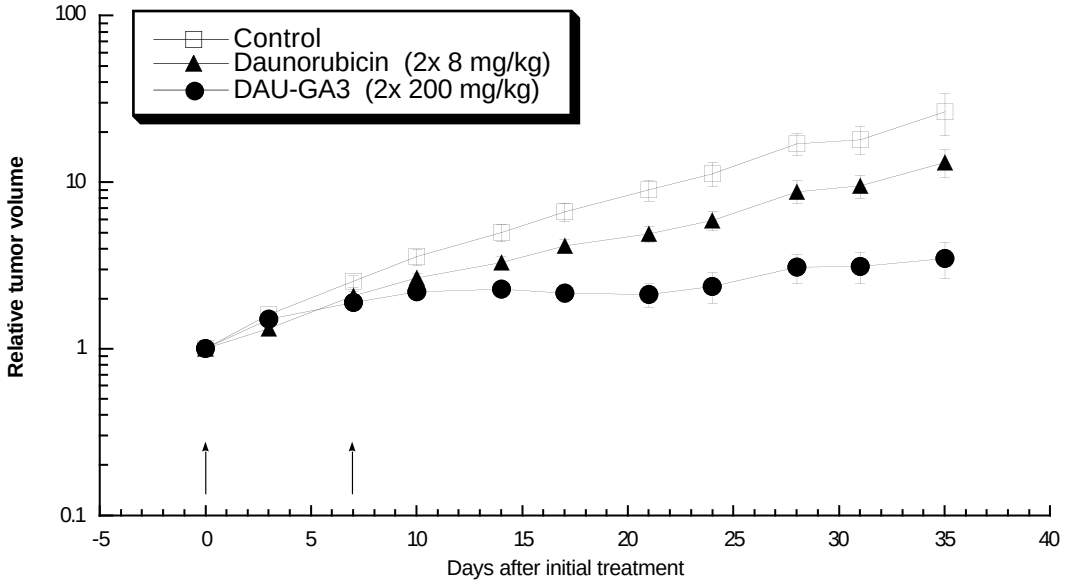


Figure 6.1 *Monotherapy using prodrug **DAU-GA3**.*

DAU-GA3 (2x 150 or 2x 200 mg/kg on day 0 and 7), was given to the mice; a second group received 2x 8 mg/kg daunorubicin on day 0 and 7 and a third group received no treatment (control group). Treatment of the mice with the prodrug **DAU-GA3** showed

a larger growth inhibition effect when compared with tumors treated with daunorubicin alone, see figure 6.1 [9].
Monotherapy of the OVCAR-3 bearing nude mice with the doxorubicin prodrug **DOX-GA3** seemed to be even more promising than the monotherapy experiments with **DAU-GA3** outlined above. When compared with the **DAU-GA3** monotherapy experiment, a larger dose of **DOX-GA3** was given to the mice because the MTD of **DOX-GA3** (2x 500 mg/kg) is higher than that of **DAU-GA3** (2x 250 mg/kg). In these experiments 2x 500 mg/kg **DOX-GA3** was given also on day 0 and 7. The second group received 2x 8 mg/kg doxorubicin (which is the MTD of doxorubicin) and the third group was not treated (control group). In the group of mice treated with **DOX-GA3** not only a growth delay but even a decrease of the tumor volume was observed after administration of the second dose of prodrug. The tumors of mice receiving doxorubicin did not reduce in volume, only a growth delay was observed when compared with the tumor-growth of in the control group. In conclusion, the effect of doxorubicin prodrug **DOX-GA3** in monotherapy experiments to treat human cancer xenografts in mice is superior to the treatment of the mice with doxorubicin itself.

6.5 Outlook: Clinical trials in cancer patients

In two recent publications Bagshawe *et al.* [10] have reported the first clinical experience with the use of an ADEPT system employing an aniline mustard prodrug in combination with a mAb-carboxypeptidase G2 conjugate (see section 1.4.3). A serious limitation of this anti-cancer therapy, however, is the development of antibodies against the bacterial carboxypeptidase G2 enzyme and immunosuppression using cyclosporin was necessary. Despite this disadvantage of the use of the non-human enzyme, Bagshawe and co-workers made a strong case for further development of the ADEPT approach of treating cancer and a number of ADEPT systems have entered into the clinic [11]. The major problems associated with ADEPT, however, originate from the necessity for use of a mAb-enzyme conjugate (main problems are: development of host antibodies, slow clearance from circulation after tumor-localization and low availability). Other prodrug activating systems have been devised to overcome the limitations imposed by the mAb-enzyme conjugate. Among these are the use of a fusion protein and the use of humanized catalytic antibodies (abzymes). For a more comprehensive overview of tumor selective prodrug activating systems, see section 1.4.
The preclinical therapeutic efficacy of our anthracycline-β-D-glucuronide prodrug/β-glucuronidase ADEPT system, simultaneously making use of the endogenous β-glucuronidase activity of necrotic tumors to activate the prodrug, has been demonstrated in mice. In this system, even without targeted β-glucuronidase activity, the β-D-glucuronide containing prodrug already possesses selective toxicity for the tumor cells (monotherapy). Additional β-glucuronidase activity can then be targeted (e.g. mAb-enzyme, fusion protein or abzyme) to enhance the efficacy of the β-D-glucuronide containing prodrug. Finally, the use of prodrugs of anthracyclines in ADEPT seem advantageous because of the lipophylicity of anthracyclines and their potential to readily enter cells. Once an anthracycline is generated from the

anthracycline-prodrug, leakage of the anthracycline into the surrounding healthy tissue or into the circulation is therefore expected to be minimal.
For the elaboration of our ADEPT system and the monotherapy approach, promising *in vivo* experiments have been performed using prodrugs **DAU-GA3** and **DOX-GA3** to treat human tumor xenografted mice. From these, prodrug **DOX-GA3** was chosen to be further evaluated in phase 1 clinical experiments [12]. As a consequence, multiple gram quantities of **DOX-GA3** are required. Because the existing synthesis of **DOX-GA3** does not allow the preparation of large amounts, a modified method to prepare this compound on a large scale is currently under investigation. The development of an anti-cancer therapy using **DOX-GA3** is financed by Pharmachemie BV Haarlem, the Netherlands.
Simultaneous to the elaboration of the encouraging future prospects of **DOX-GA3** as a tumor selective anti-cancer agent, research is continued to prepare **DOX-GA3** analogs with improved properties (e.g. blood elimination rate, activation rate, cellular uptake, cytotoxicity) for use in ADEPT and/or monotherapy. Moreover, research has started to apply the successful spacer-β-D-glucuronyl carbamate pro-moieties in the design and synthesis of prodrugs of other anti-tumor compounds (e.g. taxol).

6.6 References and notes

[1] Thesis P.H.J. Houba, Vrije Universiteit of Amsterdam, in preparation (preliminary title: Selective Chemotherapy with Glucuronidated Anthracyclines Activated by Monoclonal Antibody-β-Glucuronidase Conjugates).

[2] This is advantageous because repeated administration of the mAb-GUS conjugate/prodrug combination may prove necessary for successful treatment of patients.

[3] Houba, P.H.J.; Boven, E.; Haisma, H.J. *Bioconjugate Chem.* **1996**, *7,* 606.

[4] Houba, P.H.J.; Leenders, R.G.G.; Boven, E.; Scheeren, J.W.; Pinedo, H.M. Haisma, H.J. *Biochem. Pharmacol.* **1996**,*52*, 455. In this paper, **DAU-GB6** is referred to as "GB5".

[5] Connors, T.A.; Whisson, M.E. *Nature* **1966**, *210*, 866.

[6] Fishman, W.H.; Anlyan, A.J. *J. Biol. Chem.* **1947**, *169,* 449.

[7] Bosslet, K.; Czech, J.; Hoffmann, D. *Tumor Targeting* **1995**, *1*, 45.

[8] Mürdter, T.E.; Spreker, B.; Kivistö, K.I.; McClellan, M.; Fritz, P.; Friedel, G.; Linder, A.; Bosslet, K.; Toomes, H.; Dierkesmann, R.; Kroemer, H.K. *Cancer Res.* **1997**, *57,* 2440.

[9] Houba, P.H.J.; Boven, E.; Erkelens, C.A.M.; Leenders, R.G.G.; Scheeren, J.W.; Pinedo, H.M.; Haisma, H.J.; The Efficacy of the Anthracycline Prodrug Daunorubicin-GA3 in Human Ovarian Cancer Xenografts; submitted for publication to *Br. J. Cancer.*

[10] a] Bagshawe, K.D.; Sharma, S.K.; Springer, C.J.; Antoniw, P. *Tumor Target.* **1995**, *1,* 17; b] Bagshawe, K.D.; Regent, R.H.J. *Adv. Drug Del. Rev.* **1996**, *22,* 365.

[11] a] Jungheim, L.N.; Shepherd, T.A. *Chem. Rev.* **1994**, *94,* 1553 b] Personel communication with K. Bosslet.

[12] Bosslet and co-workers found that *in vivo* experiments employing their β-glucuronide ADEPT system in monkeys was more favorable when compared to experiments using rodents (mouse, rat); see: Bosslet, K.; Czech, J.; Hoffmann, D. *Cancer Res.* **1994**, *54,* 2151. These findings are encouraging for the translation of ADEPT from mice to humans.

Summary

This thesis is devoted to the synthesis and biological evaluation of prodrugs of anthracycline anti-tumor antibiotics designed for use in selective chemotherapy.
The severe side effects caused by conventional cancer chemotherapeutic agents arises from the lack of distinction between tumor and normal cells. Most of the available antitumor drugs are specifically toxic to proliferating cells and, therefore, tumor cells are slightly more sensitive to cytostatic agents compared with normal cells. Nevertheless, therapeutic indices of anticancer drugs are too low resulting in unacceptable damage to healthy tissue upon chemo-treatment. This has stimulated the development of a variety of approaches to improve the effectiveness and tumor cell selectivity of anticancer agents.
In this thesis the synthesis of prodrugs which can be used in the **A**ntibody **D**irected **E**nzyme **P**rodrug **T**herapy (ADEPT) concept for selective cancer treatment is described. A concise biological evaluation of the synthesized prodrugs for the ADEPT concept is given at the end of each chapter.
In the ADEPT approach for treatment of cancer (see figure S.1.), an enzyme is chemically coupled to a monoclonal antibody (mAb) which is selective for tumor associated antigens. This conjugate is administered to a cancer patient and allowed to localize selectively at the tumor site (step 1). After the mAb-enzyme conjugate is cleared from plasma and other tissue, in the second step, a relatively non-toxic prodrug is administered. This prodrug is converted to the parent cytotoxic drug by the action of the targeted enzyme.

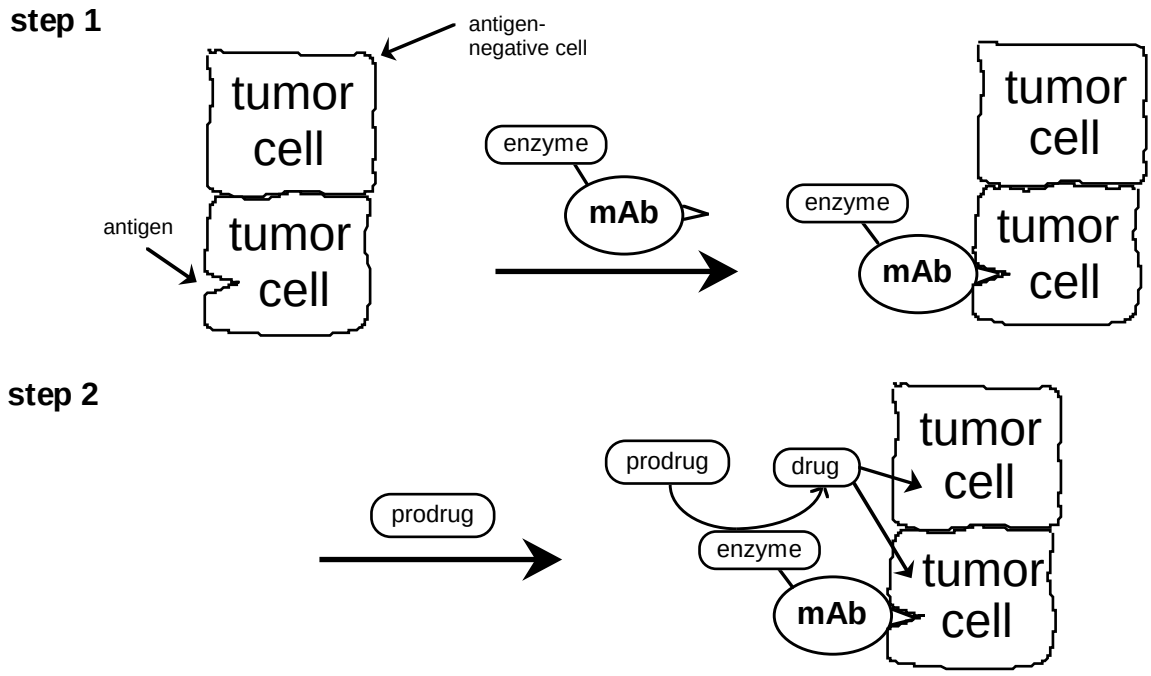
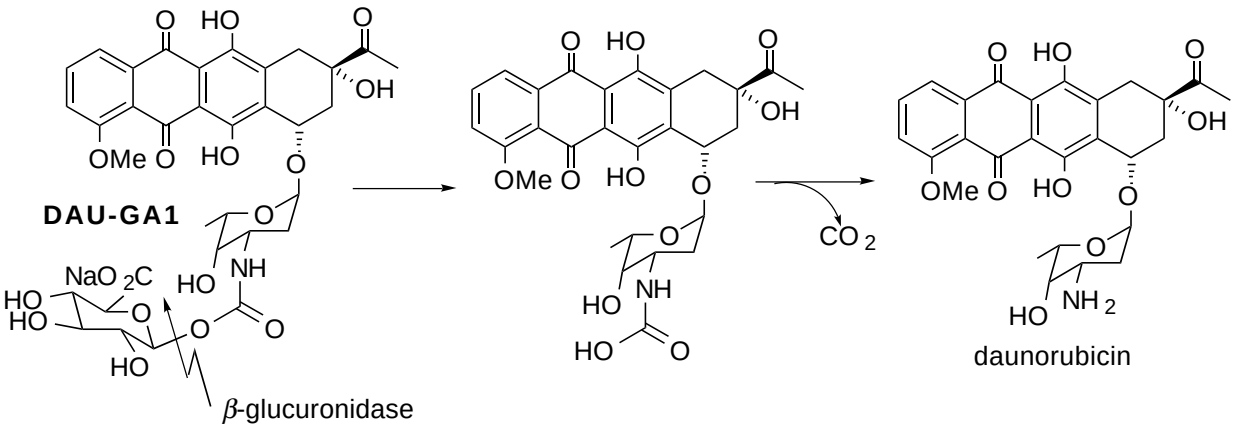


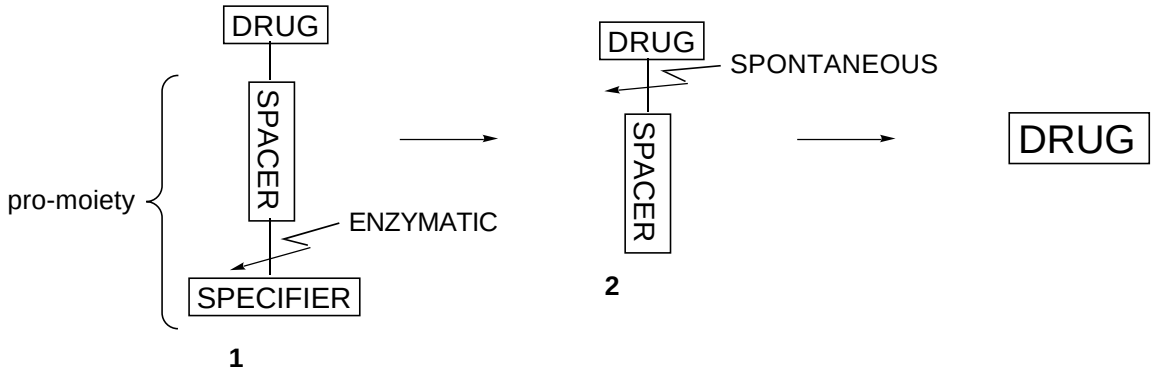
Figure S.1. *The concept of ADEPT.*

This ADEPT strategy has a number of advantages over conventional cancer chemotherapy which is outlined in more detail in chapter 1. In this introductory chapter, an overview of the literature concerning prodrugs used in ADEPT is presented.
In chapter 2, an enzyme/prodrug combination which is most promising for use in ADEPT is selected. In order to be able to select a suitable ADEPT system, polar prodrugs from daunorubicin, employing phosphate-, sulfate-, β-glucuronide-, β-glucoside- and β-galactoside groups were synthesized and subjected to a series of cytotoxicity- and enzyme-hydrolysis assays. The prodrug containing a β-glucuronyl carbamate group (**DAU-GA1**, scheme S.1.) was found to be the most promising prodrug and further research was focussed on such β-glucuronyl carbamate containing prodrugs.



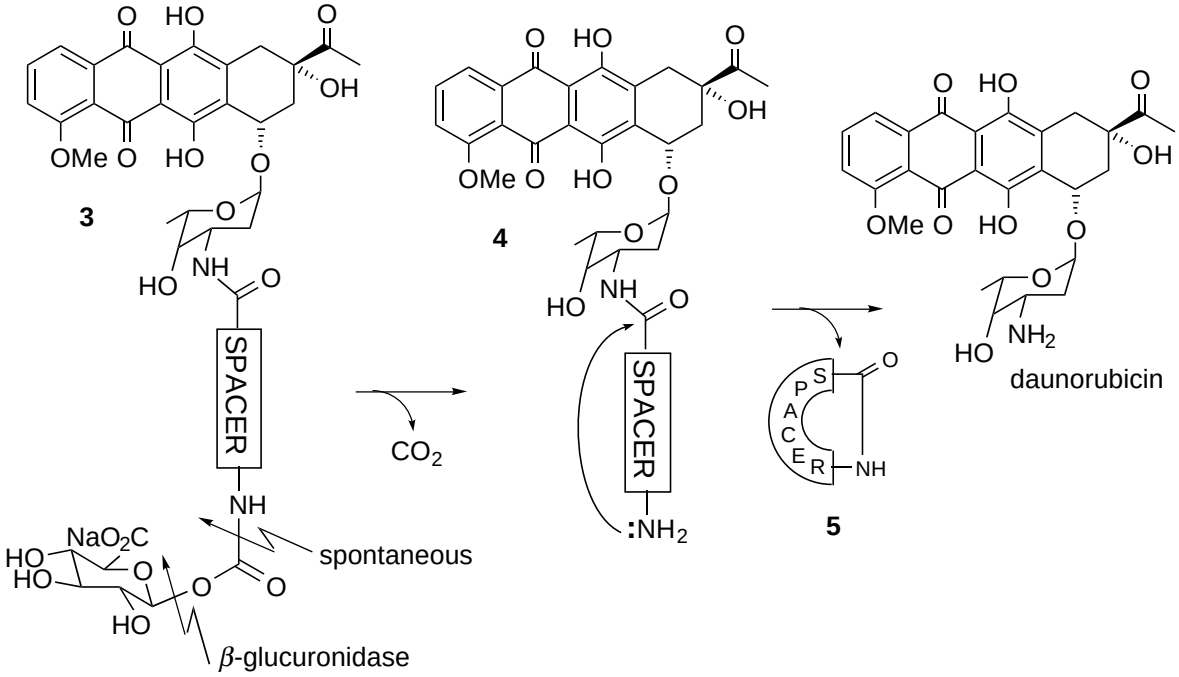
Scheme S.1. *The first β-glucuronyl carbamate containing prodrug.*

As **DAU-GA1** was activated to the parent drug daunorubicin at a rate which was considered too slow for use in ADEPT, further research was devoted to the synthesis of β-glucuronyl carbamate based prodrugs of anthracyclines containing a spacer moiety between drug and β-glucuronyl specifier group (see figure S.2.). It is conceivable that the use of a spacer facilitates enzymatic hydrolysis by decreasing steric hindrance of the drug part to the enzyme action. The results of this approach are described in chapters 3 and 4.

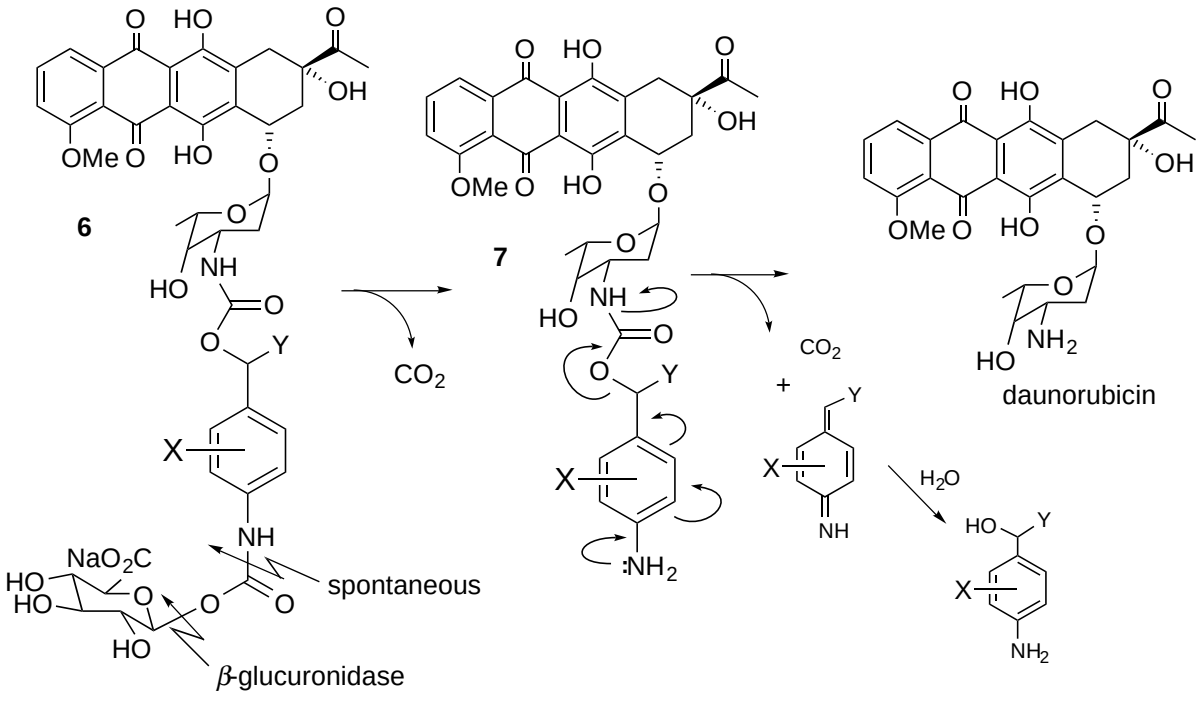


Scheme S.2. *Spacer containing prodrugs.*

Chapter 3 deals with β-glucuronyl carbamate containing prodrugs **3** derived from daunorubicin, (scheme S.3.) containing a spacer designed to immolate by a ring-closure reaction after enzymatic hydrolysis of the specifier group. Unfortunately, after hydrolysis of the glucuronyl specifier group, the spacer of drug-spacer molecules **4** did not cycloeliminate and the free drug daunorubicin was not restored.

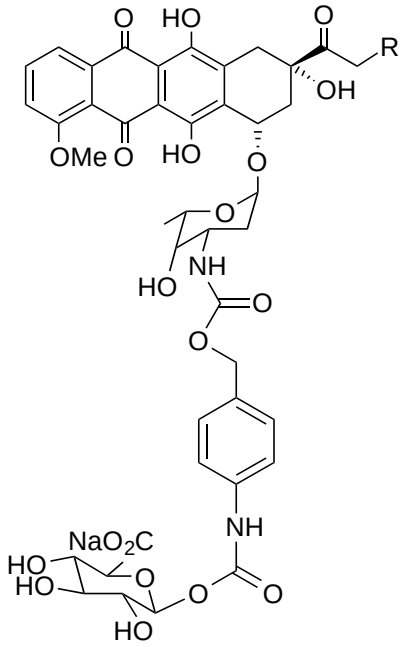


Scheme S.3. *Prodrugs containing a cycloelimination-spacer.*



Scheme S.4. *Prodrugs containing a 1,6-elimination spacer.*

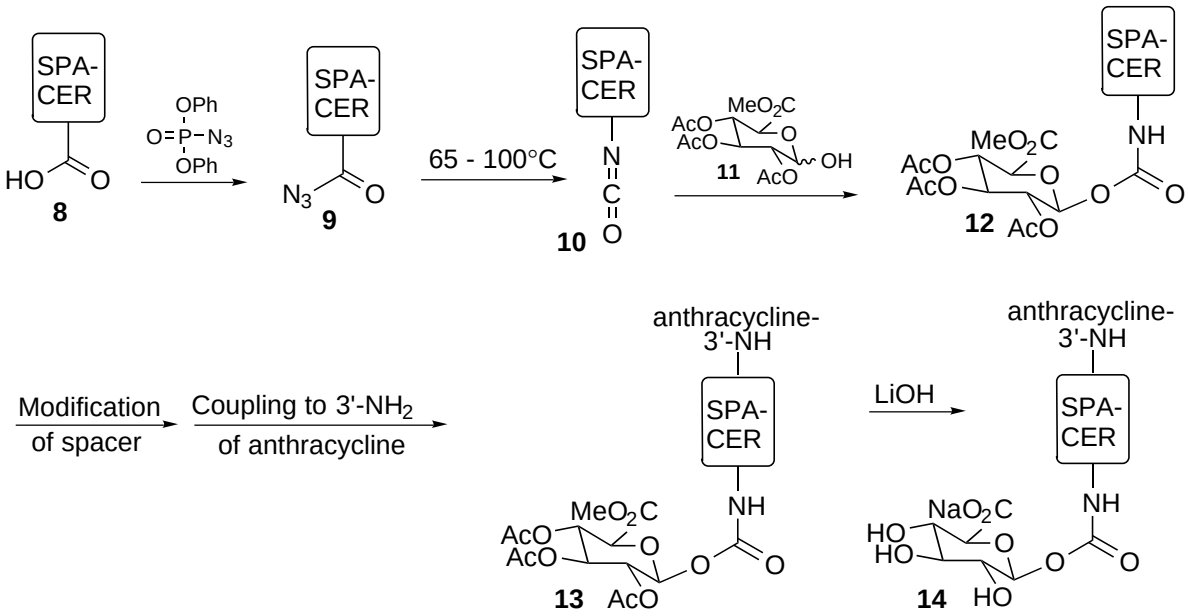
In chapter 4, *β*-glucuronyl carbamate containing prodrugs **6** (X, Y = -H, scheme S.4.) of daunorubicin and other anthracyclines are described possessing a spacer designed to immolate by a 1,6-elimination reaction after enzymatic hydrolysis of the specifier group. The spacer of these prodrugs was substituted with electron-withdrawing groups (X = -Cl, -Br) in order to facilitate enzymatic hydrolysis and serum protein binding. In addition, a prodrug containing a lipophilic tail on the spacer in order to decrease the rate of elimination from the circulation, compound **6** (X = -H, Y = -*n*-hexyl), is described. Finally, the synthesis of *β*-glucosyl- and *β*-galactosyl analogs of prodrug **6** (X, Y = -H) are presented. All the synthesized prodrugs described in chapter 4 were converted into their respective free drug upon contact with the matching enzyme. The activation rate of the prodrugs containing an electron-withdrawing group on the spacer, however, was not significantly different from that of the unsubstituted prodrug. The rates of activation the *β*-glucosyl and *β*-galactosyl based prodrugs were more than twice as low as that of the *β*-glucuronyl analog and the *n*-hexyl substituted prodrug was activated at an even slower rate. The prodrug with the *n*-hexyl substituent on the spacer and the prodrugs with an halogen atom did not have improved pharmacokinetics compared to the unsubstituted prodrug **DAU-GA3** (chart S.1.). With respect to these evaluation parameters, one prodrug of daunorubicin and the corresponding analog of doxorubicin, **DAU-GA3** and **DOX-GA3**, respectively, (see chart S.1.) were selected from all prodrugs to be studied in greater detail in *in vitro* and *in vivo* biological systems. From these prodrugs, **DOX-GA3** will be further evaluated in a phase 1 clinical trial in the near future.



DAU-GA3 (R = -H)
DOX-GA3 (R = -OH)

Chart S.1.

In the key step of the synthesis of the prodrugs described in chapter 3 and 4, the *β*-glucuronyl carbamate group was introduced by the addition reaction of the glucuronyl donor **11** (or glucosyl or galactosyl donors in case of *β*-glucosyl- and *β*-galactosyl carbamates) to a spacer isocyanate **10** (scheme S.5.). The isocyanate addition reaction led to the respective glycosyl carbamates **12** in a very high *β*-diastereoselective fashion. In chapter 5, this reaction is elaborated further. In general, isocyanates **10** were synthesized from their respective spacer- carboxylic acids **8** by a modified Curtius reaction, according to scheme S.5. In some individual cases isocyanates **10** were prepared from their respective amine hydrochlorides using phosgene or diphosgene. After modification, pro-moieties **12** were coupled to an anthracycline, resulting in protected prodrugs **13**. Deprotection of compounds **13** led to prodrugs **14**.



Scheme S.5. *General synthetic scheme for the synthesis of prodrugs of chapter 3 and 4.*

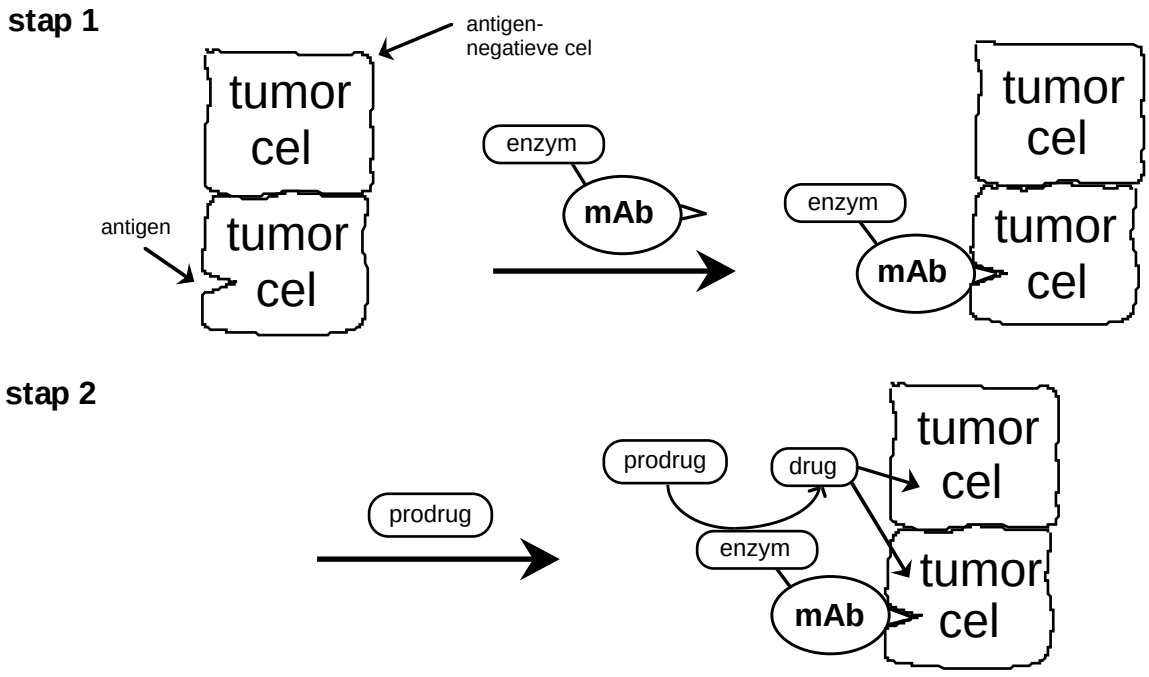
In chapter 6, a summary is given of the *in vitro* and *in vivo* experiments using the prodrugs described in this thesis as obtained at the Department of Medical Oncology, Vrije Universiteit of Amsterdam. Besides the application of the prodrugs in ADEPT also the monotherapy approach is described. In this monotherapy, no mAb-enzyme conjugate is used to activate a prodrug but the *β*-glucuronyl based prodrugs are activated by endogeneous *β*-glucuronidase present in necrotic tumor areas. Some results of monotherapy experiments using **DAU-GA3** are presented.

Concluding Remarks

The research described in this thesis has led to the development of a number of general synthetic pathways for spacer-containing *β*-glucuronide, *β*-glucoside or *β*-galactoside based prodrugs of anthracyclines. The synthesized prodrugs were tested to evaluate their applicability in ADEPT and monotherapy. Two prodrugs, **DAU-GA3** and **DOX-GA3** (chart S.1.), were chosen for further studies. Of these compounds, **DOX-GA3** was found to be the most promising prodrug for further evaluation in ADEPT and monotherapy for the treatment of cancer. This compound will be further evaluated for application in a clinical trial in the near future.

Samenvatting

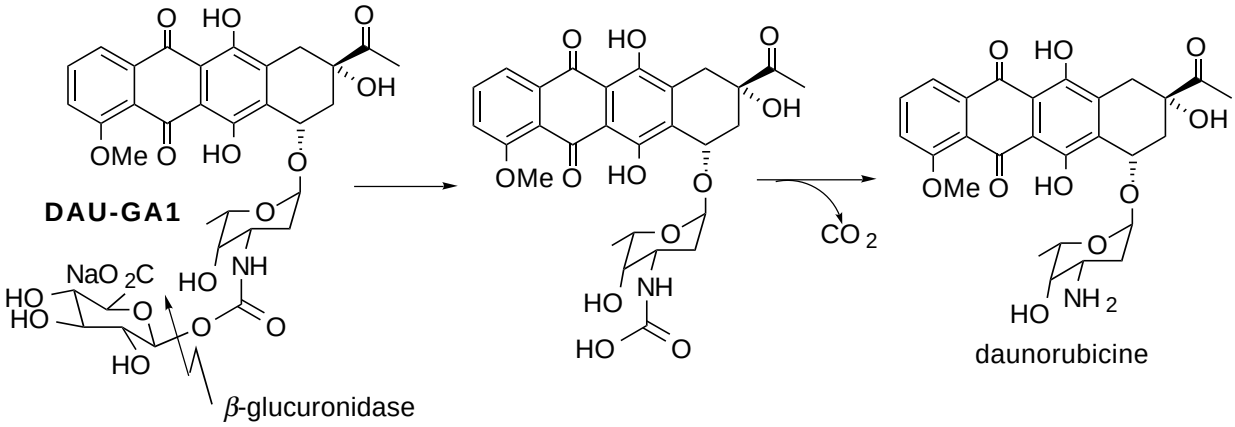
In dit proefschrift wordt de synthese en de biologische evaluatie van prodrugs van anthracycline anti-tumor antibiotica beschreven. Deze zijn ontworpen voor gebruik in selectieve chemotherapie.
De ernstige bijwerkingen van conventionele anti-kanker medicijnen worden veroorzaakt door het gebrek aan verschil tussen kanker- en gezonde cellen. De meeste anti-tumor middelen zijn giftig in het bijzonder voor snel delende cellen en daarom zijn tumor-cellen iets gevoeliger voor deze middelen dan normale cellen. Ondanks dat is de therapeutische index van anti-tumor middelen te laag en onaanvaardbare schade aan gezond weefsel is een gevolg van een chemokuur. Deze problemen hebben onderzoek gestimuleerd naar de ontwikkeling van meer selectieve anti-tumor middelen.
In dit proefschrift wordt de synthese van prodrugs beschreven die gebruikt kunnen worden in het **A**ntibody **D**irected **E**nzyme **P**rodrug **T**herapy (ADEPT) concept voor de selectieve behandeling van kanker. Een beknopte biologische evaluatie van de gesynthetiseerde prodrugs wordt gegeven aan het einde van elk hoofdstuk.
In het ADEPT concept voor de behandeling van kanker (zie figuur S.1.), wordt een enzym covalent gebonden aan een monoklonaal antilichaam (mAb). Het gebruikte mAb is selectief voor tumor-geassocieerde antigenen. Dit mAb-enzym conjugaat wordt toegediend aan een kanker patient waarna het selectief aan tumor-cellen bindt (stap 1).



Figuur S.1. *Het ADEPT concept.*

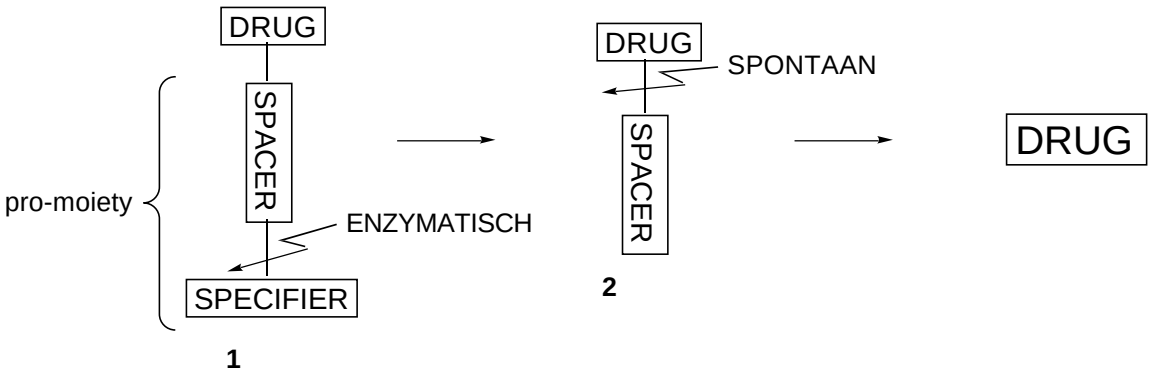
Nadat het niet gebonden mAb-enzym conjugaat uit het bloed en uit ander weefsel is verdwenen, wordt in stap 2 een relatief onschadelijke prodrug toegediend. Deze prodrug wordt omgezet tot de oorspronkelijke anti-kanker drug door het, met behulp van het antilichaam naar de tumor gebrachte enzym.

Het ADEPT concept en de literatuur over prodrugs die voor ADEPT ontworpen en gemaakt zijn, wordt in hoofdstuk 1 gehandeld.

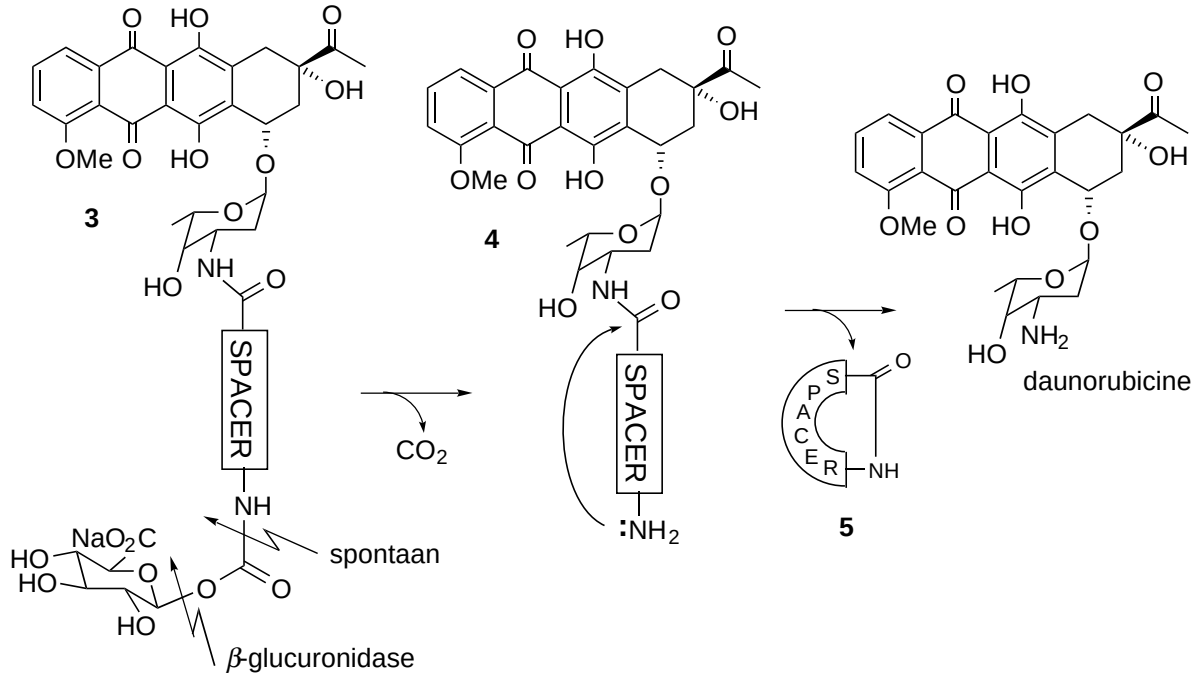


Schema S.1. *De eerste β-glucuronyl carbamaat-bevattende prodrug.*

In hoofdstuk 2 wordt het meest veelbelovende enzym/prodrug systeem voor gebruik in ADEPT gekozen. Om een keuze tussen verschillende ADEPT systemen te kunnen maken, zijn er polaire prodrugs van daunorubicine gemaakt die met het overeenkomstige enzymen geactiveerd kunnen worden. De prodrugs met fosfaat-, sulfaat-, *β*-glucuronide-, *β*-glucoside- of *β*-galactoside groepen zijn gesynthetiseerd en getest op cytotoxiciteit en enzym activerings-snelheid.
De prodrug met de *β*-glucuronyl carbamaat groep (**DAU-GA1**, schema S.1.) was het meest veelbelovend, maar werd geactiveerd tot daunorubicine met een te lage snelheid voor toepassing in ADEPT. Vervolg onderzoek werd geconcentreerd op de synthese van *β*-glucuronyl carbamaat bevattende prodrugs van anthracyclines met een spacer groep tussen drug en *β*-glucuronyl specifier (zie figuur S.2.). Er werd verwacht dat de spacer de enzymatische hydrolyse van de prodrug zou vergemakkelijken door vermindering van de sterische hindering dat het enzym van de drug ondervindt. Dit is beschreven in hoofdstuk 3 en 4.

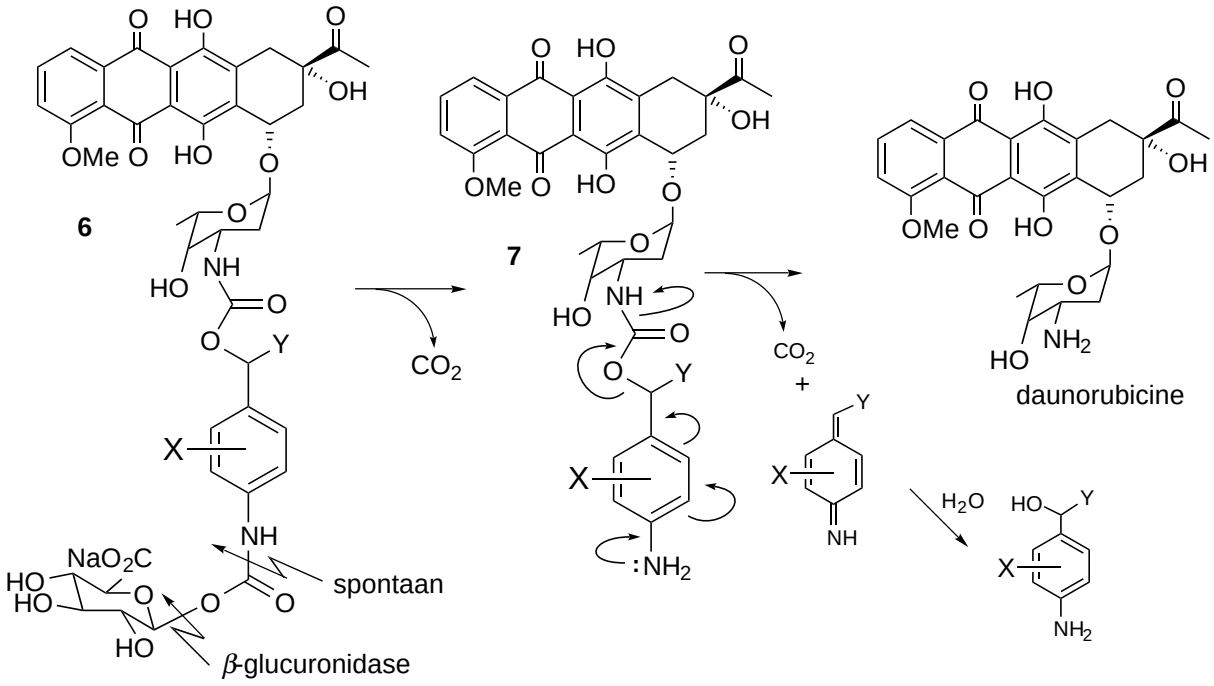


Schema S.2. *Spacer bevattende prodrugs.*



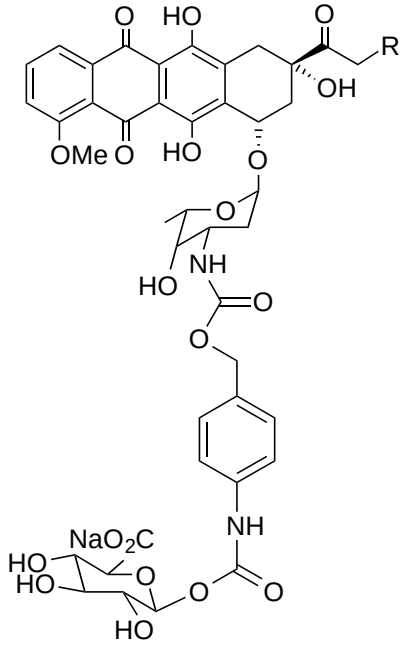
Schema S.3. *Prodrugs met een cycloeliminatie-spacer.*

In hoofdstuk 3 worden spacer-β-glucuronyl carbamaat bevattende prodrugs **3** (schema S.3.) van daunorubicine beschreven. De spacer van deze prodrugs zou, na enzymatische hydrolyse van de specifier groep, van het resulterende drug-spacer molecuul **4** moeten elimineren via een ring-sluitings mechanisme. Dit gebeurde helaas niet, drug-spacer molecuul **4** bleef intact na hydrolyse van de β-glucuronyl specifier groep en de vrije drug werd niet verkregen.



Schema S.4. *Prodrugs met een 1,6-eliminatie spacer.*

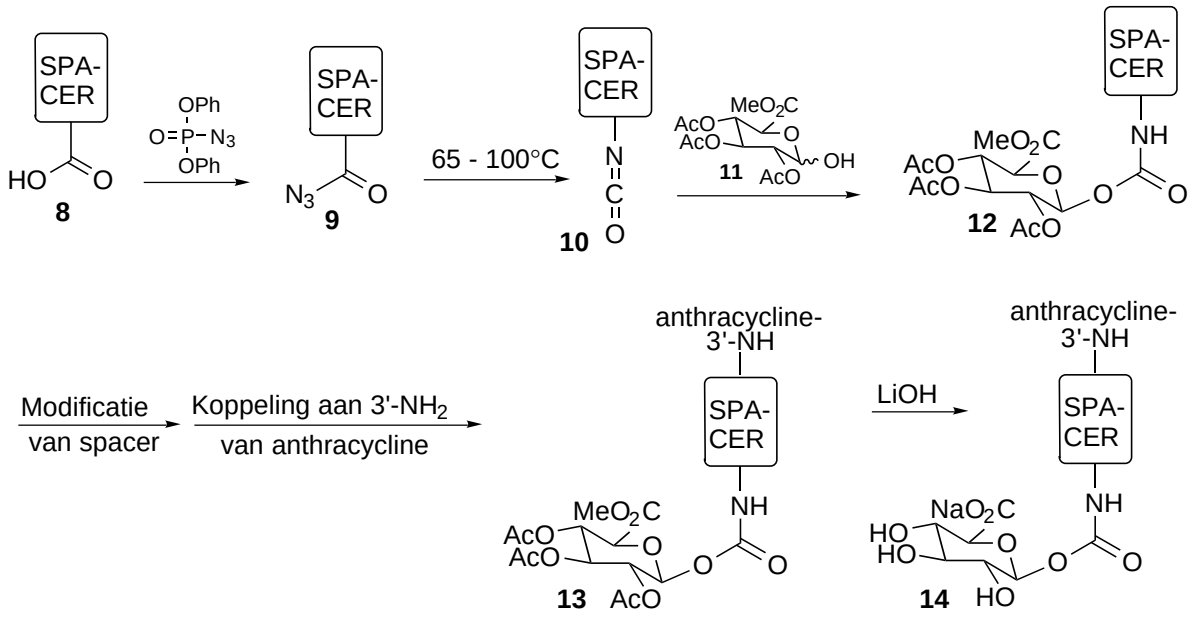
In hoofdstuk 4 worden prodrugs van daunorubicine en andere anthracyclines beschreven die een *β*-glucuronyl carbamaat groep bevatten met een andere spacer, zie prodrugs **6** in schema S.4. De prodrugs bevatten een spacer die, na enzymatische hydrolyse van de specifier groep, af kan splitsen in een 1,6-eliminatie reactie. De spacer van deze prodrugs is gesubstitueerd met electronenzuigende groepen (X = -Cl, -Br, $-NO_2$) bedoeld om de enzymatische hydrolyse te versnellen. Ook kunnen chloor- en broomaromaten aan serum-eiwitten binden. Dit kan de uitscheidings-snelheid van de prodrug verkleinen. Daarnaast is er een prodrug met een lipofiele staart gemaakt, verbinding **6** (X = -H, Y = -*n*-hexyl). met het doel de uitscheidings-snelheid te verkleinen. Ten slotte zijn *β*-glucosyl- en *β*-galactosyl analoga van prodrug **6** gemaakt. Alle prodrugs beschreven in hoofdstuk 4 werden geactiveerd tot de vrije drug door het bijbehorende enzym. De activerings-snelheid van de prodrugs met de electronenzuigende substituenten was echter niet significant beter dan dat van de ongesubstitueerde prodrug. De activerings-snelheid van de *β*-glucosyl en *β*-galactosyl bevattende prodrugs was meer dan twee keer zo laag als dat van het *β*-glucuronyl analogon. De *n*-hexyl gesubstitueerde prodrug werd zelf nog langzamer geactiveerd. De prodrug met de *n*-hexyl substituent en de prodrugs gesubstitueerd met een halogeen atoom, hadden geen verbeterde farmacokinetiek vergeleken met de ongesubstitueerde prodrug **DAU-GA3** (chart S.1.). Beoordeeld naar deze karakteristieken, waren er twee prodrugs uitgekozen die in meer detail bestudeerd zijn in *in vitro* en *in vivo* biologische systemen. Dit zijn prodrugs van daunorubicine (**DAU-GA3**) en doxorubicine (**DOX-GA3**). Van deze prodrugs is **DOX-GA3** gekozen om verder geëvalueerd te worden voor toepassing in de kliniek.



DAU-GA3 (R = -H)
DOX-GA3 (R = -OH)

Chart S1

In de belangrijkste synthese-stap van de spacer-bevattende prodrugs, wordt de *β*-glucuronyl carbamaat groep geïntroduceerd door middel van een additie reactie van glucuronyl donor **11** aan spacer isocyanaten **10** (of de corresponderende glucosyl- of galactosyl donoren in het geval van *β*-glucosyl en *β*-galactosyl carbamaten) zie schema S.5. Deze additie reactie leidt tot de corresponderende *β*-glycosyl carbamaten **12** in een zeer hoge *β*-diastereoselectiviteit. De reactie is uitgebreid beschreven in hoofdstuk 5.
Spacer-isocyanaten **10** werden in de meeste gevallen gesynthetiseerd uit spacer-carbonzuren **8** door middel van een gemodificeerde Curtius reactie volgens schema S.5. In sommige gevallen werd spacer-isocyanaat **10** gesynthetiseerd uit het corresponderende amine hydrochloride waarbij gebruik werd gemaakt van fosgeen of difosgeen. Pro-moieties **12** konden na modificatie gekoppeld worden aan een anthracycline, leidende tot de beschermde prodrugs **13**. Na ontscherming van **13** werden prodrugs **14** verkregen.



Schema S.5. *Algemeen schema voor de synthese van prodrugs in hoofdstuk 3 en 4.*

In hoofdstuk 6 wordt een samenvatting gegeven van de *in vitro* en *in vivo* experimenten waarin de beste prodrugs van dit proefschrift gebruikt worden. Deze experimenten zijn uitgevoerd op de afdeling Medische Oncologie aan de Vrije Universiteit van Amsterdam. Naast de toepassing van de prodrugs in ADEPT, is de monotherapie benadering in dit hoofdstuk beschreven. In de monotherapie benadering worden de prodrugs geactiveerd door het endogene enzym *β*-glucuronidase, dat vrijkomt uit necrotische gebieden in een tumor. Er behoeft dus in dit geval geen mAb-enzym conjugaat toegediend te worden om de prodrug te activeren. Enige resultaten van **DAU-GA3** in monotherapie experimenten zijn beschreven.

Afsluitende opmerkingen

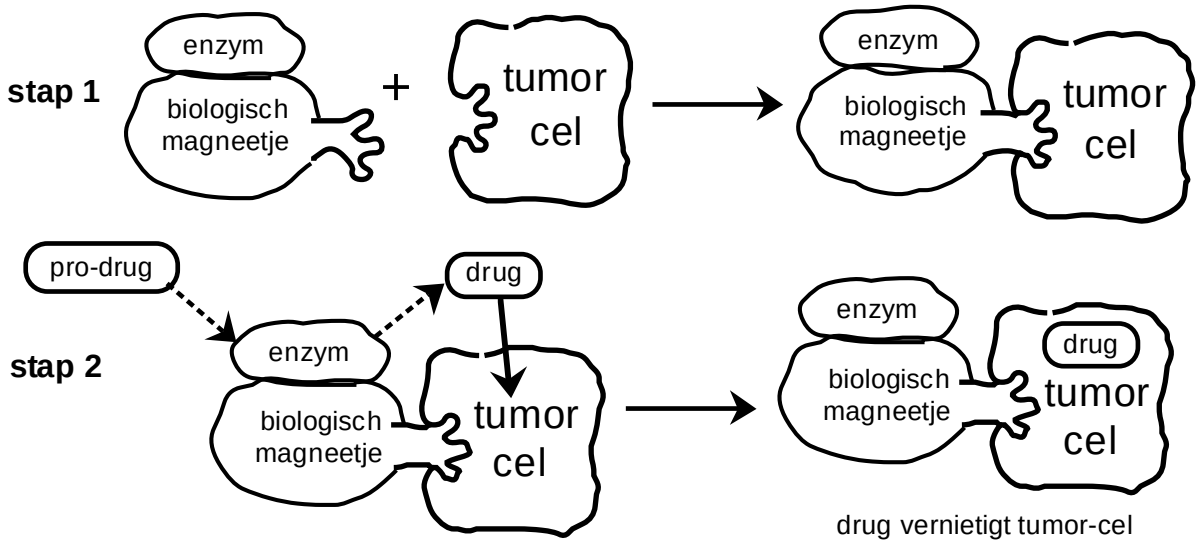
Het onderzoek beschreven in dit proefschrift heeft geleid tot de ontwikkeling van een aantal algemene synthetische procedures voor de bereiding van spacer bevattende *β*-glucuronide, *β*-glucoside en *β*-galactoside prodrugs van anthracyclines. De gesynthetiseerde prodrugs zijn getest op hun bruikbaarheid in het ADEPT concept voor de behandeling van kanker. Twee prodrugs, **DAU-GA3** en **DOX-GA3** (chart S.1.), werden geselecteerd voor uitgebreide *in vivo* studies. Van deze prodrugs was **DOX-GA3** het meest veelbelovend om verder geëvalueerd te worden in ADEPT en in monotherapy. Deze verbinding zal verder worden geëvalueerd voor toepassing in een klinische fase 1 onderzoek.

Samenvatting voor niet-chemici

Deze samenvatting is geschreven voor degenen die de rest van dit proefschrift zó ondoorgrondelijk vinden dat ze, na het lezen van het voorwoord, al direct afhaken.

Anti-tumor middelen (= cytostatica) zijn giftig speciaal voor snel delende cellen. Omdat tumor-cellen sneller groeien dan gezonde cellen, zijn tumor-cellen gevoeliger voor cytostatica dan normale cellen. Dit verschil is echter klein en de behandeling van een patiënt met een chemokuur gaat dan ook gepaard met ernstige bijwerkingen en een grote schade aan gezond weefsel. Ernstige bijwerkingen zijn: haaruitval, misselijkheid, beenmerg afbraak en vergiftiging van de hartspier. Omdat de conventionele cytostatica zo giftig zijn, kan er maar een lage dosis aan een patiënt gegeven worden. Om toch genoeg effect van de behandeling te hebben, worden er, gedurende een langere periode, meerdere doses van een lage concentratie van het anti-tumor middel toegediend. Doordat de tumor-cellen op deze manier niet in een paar keer gedood kunnen worden, is de kans groot dat tijdens zo'n lange chemokuur, tumor-cellen ontstaan die resistent zijn geworden tegen de cytostatica. De resistente cellen zullen overleven en na verloop van tijd bestaat de gehele tumor uit deze resistent cellen. De tumor is niet meer te bestrijden met de gebruikte middelen en vaak ook niet meer met andere cytostatica (= multi-drug resistentie).
Het onderzoek dat in dit proefschrift wordt beschreven, is gericht op het ontwikkelen van een anti-kanker medicijn dat alléén tumorcellen dood en gewone cellen ongemoeid laat. Het medicijn heeft dan minder bijwerkingen en kan in een hoge dosis aan de patiënt gegeven worden. Hierdoor is de kans op het ontwikkelen van multi-drug resistentie veel lager. In onderstaande figuur is het concept weergegeven waarmee geprobeerd wordt bestaande anti-tumor middelen selectief werkzaam voor tumorcellen te maken. In dit concept wordt een bestaand anti-tumor middel, bv doxorubicine, chemisch geïnactiveerd door er een inactiverend molecuul aan te koppelen. Het molecuul dat zo verkregen wordt is een zogenaamde pro-drug. Het inactiverende molecuul wordt het pro- gedeelte genoemd en het cytostaticum is de drug. Zo'n pro-drug is een niet-giftige variant van de drug en kan zonder (veel) problemen gebruikt worden. Zo'n pro-drug moet dusdanig ontworpenzijn, dat het door een van te voren geselecteerd enzym omgezet kan worden tot de oorspronkelijke giftige drug. Het enzym knipt letterlijk de inactiverende groep (het pro- gedeelte) van de pro-drug, zodat het oorspronkelijke cytostaticum weer is teruggevormd. Het cytostaticum zal vervolgens de tumor-cel doden.
In stap 1 van de behandeling (zie plaatje) wordt het bewuste enzym, dat gekoppeld is aan een een antilichaam (een "biologisch magneetje"), aan de patiënt toegediend. Het antilichaam bindt selectief aan een tumor-cel en nergens anders aan en brengt zo het enzym alleen naar de tumor-cel. Het enzym is dus alléén aanwezig bij de tumor en de pro-drug kan dus alléén maar bij de tumor geactiveerd worden.
In stap 2 wordt de pro-drug in de bloedbaan van de patiënt gebracht. Zodra de pro-drug in contact komt met het enzym, wordt het geactiveerd tot de giftige drug die direct de tumor-cel binnen zal dringen en zal doden. Op deze manier worden alleen

tumor-cellen blootgesteld aan de giftigheid van het anti-tumor middel. Deze strategie wordt het "ADEPT" concept genoemd (**A**ntibody **D**irected **E**nzyme **P**rodrug **T**herapy).



Het "ADEPT" concept voor behandeling van kanker

Het onderzoek dat in dit proefschrift wordt beschreven, is gericht op de ontwikkeling van een pro-drug die gebruikt kan worden in het ADEPT concept. Een goede pro-drug moet aan een aantal voorwaarden voldoen. De belangrijkste zijn: *i.* De pro-drug moet veel minder giftig zijn dan de oorspronkelijke drug, *ii.* de pro-drug moet door het enzym met een goede snelheid omgezet kunnen worden tot de drug (om een giftige hoeveelheid van de drug te kunnen genereren), en *iii.* de pro-drug moet gezonde cellen niet binnen kunnen dringen. Als de pro-drug een gezonde cel binnen zou dringen, dan zal hij niet meer bij de tumor-cel kunnen komen.
Tijdens het onderzoek is er gezocht naar een pro-drug die het beste voldoet aan bovenstaande voorwaarden. Hiertoe is een aantal pro-drugs ontworpen en gemaakt (= chemisch gesynthetiseerd). De pro-drugs zijn vervolgens getest op bovenstaande eigenschappen. Met behulp van deze testresultaten zijn nieuwe, verbeterde prodrugs ontworpen en gemaakt, die op hun beurt weer getest zijn. Op deze manier is er in een aantal stappen (ontwerpen-maken-testen, ontwerp bijstellen-maken-testen, etc.) een zo optimaal mogelijke pro-drug ontwikkeld. Deze pro-drug zou de juiste eigenschappen moeten beziten om tumor-cellen te doden, zonder dat dit gepaard gaat met ernstige bijwerkingen voor de patiënt, en zonder dat drug-resistentie optreed (zie boven). De meest veelbelovende pro-drug die uit het onderzoek naar voren is gekomen, zal in de nabije toekomst getest worden op patiënten in een experimentele chemokuur.

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Curriculum Vitae

Ruben Leenders werd geboren op 21 januari 1966 te Nijmegen alwaar hij in 1984 het VWO diploma behaalde op het Dukenburg College. In dat zelfde jaar werd de studie scheikunde begonnen aan de KU Nijmegen. In oktober 1990 werd het doctoraal examen behaald met als hoofdrichting organische chemie in de werkroep van Dr. J.W. Scheeren onder begeleiding van Drs J. Keysers. Als nevenrichting werd klinische chemie gekozen op de afdeling neurologie in de groep van Dr. R.A. Wevers en Dr. J.G.N. de Jong.
Van november 1990 tot en met februari 1993 werd er een pilotstudie verricht naar selectieve chemotherapie. Eerst in het kader van de vervangende militaire dienst en later als toegevoegd onderzoeker. In deze tijd werd er een project aanvraag ingediend bij het Koningin Wilhelmina Fonds voor Kankerbestrijding (KWF). Dit project werd toegekend en van maart 1993 tot en met maart 1997 was de auteur van dit proefschrift AIO op dit onderwerp, onder dagelijkse begeleiding van Dr. J.W. Scheeren.